AN INVESTIGATION OF THE CARDIOTOXIC, INFLAMMATORY AND IMMUNOLOGIC RESPONSES OF HORSES TO RATTLESNAKE VENOM INCLUDING DEVELOPMENT OF A FLUORESCENT ELISA FOR DETECTION OF RATTLESNAKE VENOM IN EQUINE BIOLOGICAL SAMPLES

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AN INVESTIGATION OF THE CARDIOTOXIC, INFLAMMATORY AND IMMUNOLOGIC RESPONSES OF HORSES TO RATTLESNAKE VENOM INCLUDING DEVELOPMENT OF A FLUORESCENT ELISA FOR DETECTION OF RATTLESNAKE VENOM IN EQUINE BIOLOGICAL SAMPLES

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"In all things God works for the good of those who love Him…" ~Romans 8:28 It began in Dalhart, Texas when my horses were rattlesnake bitten. It was difficult to see how good could come from such tragedy but God gave me the means to carry out this project and all glory goes to Him! The blessings that allowed me to reach this destination follow: My major advisor, Dr. Ownby, my journey would've ended prematurely without you. I'm forever changed by your fingerprints on my life. My mentor, colleague and friend, Dr. Holbrook, how could've God made 2 people's paths intersect more perfectly? I'm forever grateful for the internist you created! Dr. Dianne McFarlane – Thank you for challenging me and being an example of a successful researcher! Dr. Clint Krehbiel – I could count on your kind, gentle, and honest spirit for encouragement. Dr. Reed Holyoak – Thanks for believing I'm a superstar! I hope I can be all you believe I am! My veterinary students, interns and residents that took less so I could give this more. OSU faculty/staff that helped and encouraged me. The clinics that collected samples, this wouldn't exist without you!

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Abstract: Clinical outcomes following rattlesnake bites in horses are widely variable and reasons for this variability are unknown. Being able to quantify venom dose could be helpful in further investigating the effects of rattlesnake venom in the horse. We hypothesized that ELISA techniques could be used to detect venom in equine biological samples. A double sandwich fluorescent ELISA was developed to detect venom in urine and at the bite site of horses with a clinical diagnosis of rattlesnake bite. Venom was successfully detected in equine biological samples using the fluorescent ELISA.

We hypothesized that rattlesnake bitten horses frequently experience cardiac damage. In order to detect both myocardial cell injury and electrical dysfunction, cardiac troponin I (cTnI) and electrocardiography were used to document cardiac damage in naturally envenomated horses. Twenty horses with clinical diagnosis of snake bite were included. Serum and plasma were collected at selected intervals. Holter monitors (Zymed®, Philips) were placed at presentation, 1 week and 1 month post presentation. Plasma was assayed for cTnI using a fluorometric assay (Stratus CS®, Dade Behring). A significant number of horses had elevated cTnI ($p<0.05$) at one or more time points. Holter readings were available for 20 horses and revealed arrhythmias or tachycardia in 14 horses.

We further hypothesized that increased TNF-α concentration could result in cardiac damage. Serum samples were assayed for TNF-α using a commercial assay (Endogen). There was a positive correlation between cTnI and TNF-α ($p<0.02$). We hypothesized that pre-existing anti-venom antibodies would be protective against cardiac damage. Antibody titers to *Crotalus atrox* were measured at presentation, 1 week and 1 month. None of the horses had pre-existing antibody titers and no correlations were made between cardiac damage and antibody titers.

Finally, we hypothesized that horses would produce anti-venom antibodies when vaccinated with a rattlesnake toxoid vaccine that would be comparable with natural envenomation. Naturally envenomated horses had significantly higher antibody titers than vaccinated horses ($p<0.004$).

A model of rattlesnake venom induced cardiotoxicity is needed to further investigate the causes and potential treatments of cardiac damage following rattlesnake envenomation.

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CHAPTER I

INTRODUCTION

The study of rattlesnake envenomation is a complicated task for many reasons. Rattlesnakes vary in their appearance, with even the existence of rattleless rattlesnakes, and there is no single identifying feature common to all rattlesnakes (Tu 1982). Rattlesnake nomenclature can be confusing when scientific and common names are interchanged (Table 1). The toxic effects of their venoms vary amongst genera and even within the same species (Tu 1982). Venom yield and toxicity varies with age of the snake (Tu 1982). Snakes within the same species that are located in different geographical regions may have differences in their venom (Tu 1982). Even snakes within the same species located within the same geographical area can have seasonal variations in their venoms (Tu 1982). Some toxins are common to all rattlesnake venoms while others are found only in certain species and still others remain undefined (Tu 1982). There have been documented changes in patterns of toxicity in snakes from certain geographical regions over time (Tu 1982). Bites from the Mojave rattlesnake (*Crotalus scutulatus scutulatus)* in Southern California, for example, did not historically manifest clinically with hemorrhage and tissue necrosis. However, in 2012 a case with hemorrhage and tissue necrosis was reported (Bush, Teacher et al. 2012). There is speculation that rattlesnake venoms are changing and changes could be due to interspecies breeding, for example the presence of Mojave toxin in the southern

pacific rattlesnake (*Crotalus viridis helleri*); however, this theory is controversial (Julius, Kaelble et al. 2012).

In order to better understand the complicated clinical picture that results from rattlesnake envenomation in horses it is helpful to have a working knowledge of individual venom components and their effects on the entire body. Since there is a paucity of research looking at the effects of rattlesnake venom in the horse, it is pertinent to review the literature in humans as well as other veterinary species. With this knowledge it is possible to narrow our focus to the effects of rattlesnake venom on the heart. These effects can be assessed by measuring cardiac troponin I as well as electrocardiography.

It is useful to examine the body's response to rattlesnake envenomation. Tumor necrosis factor alpha has been examined as a mediator of cardiotoxicity following Viperid envenomation (Szold, Ben-Abraham et al. 2003). This cytokine has also been examined in its role in cardiac disease in humans (Mann 2001). It's role in cardiac damage following rattlesnake evenomation has not been reported. The humoral response to snake venom has been studied (Da Silva and Tambourgi 2011). An understanding of the immunologic response is important for the examination of the effects of rattlesnake envenomation as well as potential treatments.

Finally, the study of rattlesnake venom often involves the need for venom detection. Snake venom has been detected in biological samples since 1902(Lamb 1902). A review of the evolution of the techniques used for venom detection is useful in determining which assay is best suited for an individual experiment.

Table 1. Common and scientific names of venomous snakes mentioned in this dissertation.

Scientific Name	Common Name
Crotalus adamanteus	Eastern Diamondback Rattlesnake
Crotalus atrox	Western Diamondback Rattlesnake
Crotalus horridus horridus	Timber Rattlesnake
Crotalus scutulatus scutulatus	Mojave Rattlesnake
Crotalus viridis viridis	Prairie Rattlesnake
Crotalus viridis helleri	Southern Pacific Rattlesnake
Crotalus durissus terrificus	South American Rattlesnake
Crotalus horridus atricaudatus	Canebrake Rattlesnake
Sistrurus miliarius streckeri	Pigmy Rattlesnake
Agkistrodon contortix lacintus	Southern Copperhead
Agkistrodon piscivorus	Eastern/Western Cottonmouth
Bothrops atrox	Common Lancehead
Vipera aspis	European Viper

CHAPTER II

REVIEW OF THE LITERATURE

General Toxic Effects of Rattlesnake Venom

Some clinical signs of rattlesnake envenomation are similar regardless of the species encountered; however, others are more specific to a certain species of snake. Rattlesnake venom has two primary purposes; immobilize prey and digest prey. Rattlesnakes strike their prey and then wait for it to become immobilized or dead prior to swallowing it whole. Enzymes and proteins important in immobilization vary among species of rattlesnakes. The Mojave rattlesnake (*Crotalus scutulatus scutulatus*), Timber rattlesnake (*Crotalus horridus horridus*), and South American rattlesnake (*Crotalus durissus terrificus*) all possess a neurotoxin. The Mojave toxin can cause a flaccid paralysis; however, weakness and paralysis are not commonly seen following Mojave rattlesnake envenomation (Holstege, Miller et al. 1997). This toxin is thought to work by blocking the calcium channels in the presynaptic motor neuron at neuromuscular junctions (Holstege, Miller et al. 1997). This inhibits the release of acetylcholine preventing the activation of the acetylcholine receptor on skeletal muscle thus preventing muscle contraction (Holstege, Miller et al. 1997). Effects of Mojave toxin experimentally are greatest on the motor axon terminals of the diaphragm which could lead to respiratory paralysis (Gopalakrishnakone, Hawgood et al. 1980). The primary neurotoxic sign seen with timber rattlesnake envenomation

is myokymia, a type of muscle fasciculation that resembles a wave or worm-like movement below the skin. The proposed mechanism also involves calcium channels at the presynaptic neuromuscular junction (Holstege, Miller et al. 1997).

Severe hypotension may result in venom induced shock and immobilization. Severe hypotension is multifactorial. A myocardial depressor protein has been demonstrated in western diamondback rattlesnake venom which could directly result in hypotension (Bonilla and Rammel 1976). Rattlesnake venom contains kininogenases which act on plasma globulins to form bradykinins (Hudelson and Hudelson 1995), potent vasodilators that can result in profound hypotension. Bradykinins can stimulate the body's natural phospholipase A2 resulting in the production of prostaglandins and thromboxane A2 (Hudelson and Hudelson 1995). Prostaglandins E2 and I2 cause vasodilation which results in decreased systemic arterial pressure and contributes to hypotension (Hudelson and Hudelson 1995). Prostaglandins can also cause severe congestion in the lungs, increased vascular permeability and hemorrhage (Hudelson and Hudelson 1995). Indomethacin, a cyclooxygenase inhibitor, has been shown to improve Mojave rattlesnake venom induced hypotension in a mouse model suggesting the role of prostaglandins in venom induced hypotension (Tu 1982). Large amounts of fluid may be lost in acute envenomation resulting in hypotension. Fluid losses are attributed to third space losses secondary to severe endothelial damage, vomiting and hemorrhage (Hudelson and Hudelson 1995). A lethal factor in *Crotalus* venom has been shown to cause lysis of plasma membranes resulting in microangiopathic vascular permeability which allows plasma proteins and red blood cells to leak into the surrounding tissues (Ownby, Bjarnason et al. 1978). This extravascular fluid loss can lead to volume depletion and hypoperfusion followed by hemoconcentration, lactic acidosis, and hypovolemic shock.

Another contributor to venom induced hypotension is blood pooling. Crotalidae venom has been shown to cause pooling of blood in the hepatosplanchnic vasculature of dogs and in the

lungs of cats (Peterson and Meerdink 1989). Victims may have an altered respiratory rate, pulmonary edema, and cyanosis (Peterson 2006). Cardiac perfusion will suffer with prolonged or untreated hypotension resulting in a further decrease in cardiac output. Cardiac arrhythmias may be seen (Hackett, Wingfield et al. 2002; Willey and Schaer 2005; Peterson 2006).

Tissue necrosis is an important digestive component for snakes that swallow their prey whole and is a common clinical manifestation secondary to rattlesnake bite (Tu 1982). There are several venom components that likely contribute to tissue necrosis (Table 2). Each of these components affects the tissue in a different manner, breaking it down to allow the further penetration and dissemination of venom (Singletary, Rochman et al. 2005). Some directly cause tissue damage while others have indirect effects. Venom metalloproteinases (VMPs) cause local myonecrosis and skin damage as well as hemorrhage and systemic inflammation (Holstege, Miller et al. 1997). These VMPs convert pro-tumor necrosis factor α to its active form. Tumor necrosis factor α (TNF-α) then stimulates the production of host metalloproteinases (HMPs) that degrade extracellular matrix proteins resulting in tissue damage (Tanen, Ruha et al. 2001). HMPs also cleave pro-TNF-α and result in a vicious cycle of inflammation (Holstege, Miller et al. 1997). Venom hyaluronidase and collagenase lead to deeper venom penetration through connective tissue (Peterson and Meerdink 1989). Hyaluronidase decreases connective tissue viscosity by catalyzing the cleavage of internal glycoside bonds and mucopolysaccharides while collagenases digest collagen (Peterson and Meerdink 1989). There are likely many other toxins that have yet to be identified that contribute to local tissue necrosis and swelling.

Hemorrhage is a common clinical sign following envenomation and the mechanisms vary among species (Tu 1982; Singletary, Rochman et al. 2005). Red blood cell leakage and edema occur at the bite site, and possibly systemically, secondary to damage caused by hemorrhagic toxins to the capillary endothelial cells and basement membrane of the vessel walls (Singletary, Rochman et al. 2005).

Coagulopathies are also a very common feature of rattlesnake envenomation, documented in 35-50% of cases depending on the species (Singletary, Rochman et al. 2005). Thrombocytopenia is probably the most common manifestation but fibrinolysis and disseminated intravascular coagulation have also been reported (Singletary, Rochman et al. 2005). The cause of venom induced thrombocytopenia is not well understood; however, there is at least one toxin in *Crotalus horridus* venom, crotalocytin, which has a direct effect on platelets causing platelet aggregation and the release of platelet ATP (Odeleye, Presley et al. 2004).

Table 2: Rattlesnake Venom Components (Modified from Singletary et.al. 2005)

Venom Components
Metalloproteinases
Arginine ester hydrolase
Thrombin-like enzyme
Collagenase
Hyaluronidase
Phospholipase A_2
Phospholipase B
Phospholipase C
Lactate Dehydrogenase
Phosphomonoesterase
Phosphodiesterase
Acetylcholinesterase
RNase
DNase
5'-Nucleotidase
Nicotinamide adenine dinucleotide nucleotidase
$_{L}$-Amino acid oxidase
Myotoxin a
Crotamine
Crotoxin
Mojave toxin
Viridotoxin

Clinical Signs in Humans Following Rattlesnake Envenomation

The clinical presentation of humans following rattlesnake bite can vary from mild swelling only to severe swelling, hypotension and death (Singletary, Rochman et al. 2005). The most common manifestations are pain and swelling at the site of the bite, nausea with or without vomiting, sweating, hypotension, hemorrhage and coagulopathy (Tu 1982; Singletary, Rochman et al. 2005). Other clinical signs associated with rattlesnake bite are blistering around bite site, edema, weakness, numbness or tingling, tachycardia, muscle fasciculations, unusual metallic taste, paresthesia, diplopia, dysphagia, salivation, hyporeflexia, respiratory depression, paralysis, fecal or urinary incontinence, hematuria and syncope (Juckett and Hancox 2002; Morgan, Blair et al. 2006; Norris, Wilkerson et al. 2007). While thrombocytopenia and coagulopathy are very common, severe bleeding is uncommon and hemorrhage is most commonly localized to the area of envenomation (Ruha and Curry 2009). However, massive gastrointestinal hemorrhage has been reported following rattlesnake envenomation (Ruha and Curry 2009). Compartment syndrome is uncommonly reported in humans following rattlesnake bite (Hardy and Zamudio 2006). Anaphylaxis, although more commonly reported with anti-venom administration, has been reported secondary to rattlesnake bite (Ryan and Caravati 1994; Tanen, Ruha et al. 2001). It typically develops in individuals that have been previously bitten but has been reported in individuals with no previous rattlesnake exposure (Nordt 2000; Washington and Ruha 2007). Local infection at the bite site is rare in people but can occur (Nishioka, Jorge et al. 2000). A syndrome termed venom lysis syndrome has been used to describe death due to hyperkalemia following experimental envenomation due to massive tissue necrosis prior to the development of any other hematological abnormalities developing in pigs (Meggs, Courtney et al. 2007). This syndrome has not been documented in people or veterinary species following natural envenomation. Neurotoxicity and rhabdomyolysis are reported in humans following Mojave rattlesnake envenomation (Kashani and Lovecchio 2006). The neurotoxic signs range from

cranial nerve deficits such as ptosis to profound weakness that can result in the need for mechanical ventilation (Kashani and Lovecchio 2006). These signs of neurotoxicity and rhabdomyolysis can be delayed following envenomation (Kashani and Lovecchio 2006). Severe rhabdomyolysis may complicate the clinical presentation by causing acute renal failure. Neurotoxicity and renal failure are much more common following South American rattlesnake (*Crotalus durussis terifficus)* envenomation (Silveira and Nishioka 1992) with renal failure being the primary cause of death following South American rattlesnake envenomation (Pinho, Zanetta et al. 2005). Renal failure has been described after envenomation by other species of rattlesnakes as well (Ahlstrom, Luginbuhl et al. 1991; Cruz and Alvarez 1994). Mentation changes may also be present with dullness and slurred speech (Buchanan, Phillips et al. 2008). It is unusual for humans to experience respiratory compromise secondary to rattlesnake envenomation but respiratory compromise as well as rapid airway loss have been reported (Hinze, Barker et al. 2001; Kerns and Tomaszewski 2001; Brooks and Graeme 2004; Buchanan, Phillips et al. 2008). Pregnant women seem to be at a higher risk of severe complications to rattlesnake envenomation (Langley 2010). The fetal death rate post envenomation may approach 20% while maternal death rate is approximately 4-5% compared to 0.06% in men and non-gravid women (Weinstein, Dart et al. 2009; Langley 2010). It is unusual for people to have ophthalmic exposure to rattlesnake venom as they don't typically project venom like other species of snakes such as the spitting cobra; however, ophthalmic exposure has been reported and resulted in local irritation and pain (Johnson 2009).

Clinical Signs in Veterinary Species Following Rattlesnake Envenomation

The literature documenting the effects of rattlesnake envenomation in veterinary species is not extensive; however, the clinical presentation of rattlesnake envenomation in veterinary species is reported to be similar to that of humans with the exception of mortality. Mortality is higher in dogs (1-30%) (Hackett, Wingfield et al. 2002), camelids (40-58 %) (Belknap 1994;

Dykgraaf, Pusterla et al. 2006), and horses (9-25 %) (Fielding, Pusterla et al. 2011; Dickinson, TraubDargatz et al. 1996) than in humans (0.06%) (Weinstein, Dart et al. 2009). Cats seem to be more resistant to pit viper venom than dogs on a milligram of venom per kilogram of body mass basis (Peterson 2006). In humans, some consider bites to the head more serious than those to the extremities due to an increased vascularity in this area (Weaver, Stroup et al. 1991). Head bites are more common in dogs, horses and camelids; therefore, while respiratory compromise is rare in humans, it occurs commonly in these species due to upper airway obstruction (Peterson and Meerdink 1989; Brooks and Graeme 2004; Dykgraaf, Pusterla et al. 2006). Blistering in the area of the bite, although common in people, is rarely observed in domestic animals (Harned 1982). Fever is more commonly reported in the veterinary species than people (Dickinson, TraubDargatz et al. 1996; Julius, Kaelble et al. 2012). Dogs are similar to humans in that they often experience gastrointestinal signs with ptyalism, vomiting and diarrhea; however, reports of colic, gastrointestinal reflux or diarrhea are less prevalent in horses following rattlesnake envenomation (Mansfield 1984; Hudelson and Hudelson 1995; Dickinson, TraubDargatz et al. 1996). Laminitis has been reported in horses bitten by a rattlesnake (Dickinson, TraubDargatz et al. 1996). Neurologic symptoms secondary to rattlesnake envenomation have been reported in dogs and cats (Julius, Kaelble et al. 2012). According to one study, cats may be more likely to develop neurologic symptoms following envenomation (Julius, Kaelble et al. 2012). Neurologic signs exhibited by dogs and cats include altered mentation, ambulatory or nonambulatory tetraparesis, extensor rigidity, conscious proprioceptive deficits, ataxia, and decreased spinal reflexes (Julius, Kaelble et al. 2012). There are no reports of neurologic deficits in horses secondary to rattlesnake envenomation although it is likely that they occur. Neurologic deficits are reported in cattle experimentally inoculated with *Crotalus durissus terifficus* venom (Graca, Peixoto et al. 2008). Echinocytosis is reported in humans, cats, dogs and horses with rattlesnake bite (Brown, Meyer et al. 1994; Walton, Brown et al. 1997; Willey and Schaer 2005). Renal failure is rarely reported in veterinary species (Willey and Schaer 2005). In stark contrast to

humans, renal failure did not occur in dogs envenomated by *Crotalus durissus terifficus*. Some urinalysis changes and increases in creatinine were reported in only a few horses bitten by *Crotalus viridis viridis* (Dickinson, TraubDargatz et al. 1996).

Cardio-specific Abnormalities Following Rattlesnake Envenomation (All Species)

Evidence of cardiac damage following rattlesnake bite is not commonly reported in humans (Rudolph, Neal et al. 1995). Electrocardiogram changes have been sparsely reported following rattlesnake envenomation with cardiac troponins either not measured or normal (Cole 1996; Buchanan, Phillips et al. 2008). Myocardial necrosis has been reported in an individual bitten by *Crotalus horridus atricaudatus* (canebrake rattlesnake) (Kitchens, Hunter et al. 1987); however, cardiac damage was not found in a group of children bitten by *Crotalus durrisus terrificus* (Cupo, de Azevedo-Marques et al. 2003). While cardiotoxicity does not appear to be a major component of rattlesnake envenomation in people, cardiac damage subsequent to rattlesnake envenomation is frequently reported in dogs and horses (Dickinson, TraubDargatz et al. 1996; Lawler, Frye et al. 2008; Mansfield 1984). It has also been documented in experimentally envenomated cats (Goddard, Schoeman et al. 2011). Evidence of myocardial damage included increased cardiac troponin I, electrocardiographic abnormalities (Goddard, Schoeman et al. 2011) and both gross and microscopic pathological changes (Dickinson, TraubDargatz et al. 1996; Lawler, Frye et al. 2008). Electrocardiogram abnormalities have been recorded in 42-47% of dogs bitten by the eastern diamondback rattlesnake (*Crotalus adamanteus*) (Hackett, T.B., W.E. Wingfield et al. 2002, Willey and Schaer 2005). Abnormalities were most commonly ventricular in origin but atrial premature contractions were also reported (Willey and Schaer 2005). Dogs experimentally envenomated with venom from the South American rattlesnake did not show electrocardiographic abnormalities (de Sousa-e-Silva, Tomy et al. 2003). Arrhythmias, increased cardiac troponin I, congestive heart failure and gross and microscopic pathologic abnormalities have all been documented in horses bitten by rattlesnakes (Dickinson,

TraubDargatz et al. 1996; Lawler, Frye et al. 2008; Fielding, Pusterla et al. 2011). The occurrence of these abnormalities varies widely between studies. Horses bitten by the prairie rattlesnake had a high frequency of cardiac abnormalities with 59% having tachycardia and 31% having persistent tachycardia, 16% having debilitating heart disease, and 22% having cardiac lesions either on echocardiography or necropsy (Dickinson, TraubDargatz et al. 1996). In contrast, only one horse out of 58 horses bitten in Northern California showed any signs of cardiotoxicity (atrial fibrillation) (Fielding, Pusterla et al. 2011). There are different species of rattlesnakes in these two study areas as the Prairie rattlesnake is not endemic in Northern California (Tu 1982).

Markers of Cardiac Damage – A General Overview

Cardiac troponins have been studied in humans and many veterinary species including dogs, pigs, horses, cats, cattle, sheep and goats (Serra, Papakonstantinou et al. 2010; Tharwat, Al-Sobayil et al. 2012). Cardiac troponin I is a sensitive marker of myocardial injury (Rajappa and Sharma 2005) that has not been found in tissues other than the heart (Bodor, Porterfield et al. 1995); therefore, it is the preferred marker for the diagnosis of myocardial injury (Rajappa and Sharma 2005). Cardiac troponins have been successful indicators of multiple kinds of cardiac disease including congestive heart failure, cardiomyopathy, ventricular arrhythmias, pericardial effusion, valvular disease, subaortic stenosis, infarction, third-degree heart block, endocarditis, and white muscle disease (Serra, Papakonstantinou et al. 2010). They have also been used to detect myocardial damage in multiple infectious disease states, sepsis, endotoxemia, chemotherapy toxicity and viper envenomation (Serra, Papakonstantinou et al. 2010). Myocardial damage from a variety of other causes has been documented using cardiac troponins including cardiac trauma, gastric dilatation volvulus, cardiac ‘pacing’, respiratory disease, heat stroke, extreme exercise, pancreatitis, neoplasia, severe colic, downer cow syndrome, pregnancy toxemia and renal failure (Serra, Papakonstantinou et al. 2010; Tharwat, Al-Sobayil et al. 2012).

Cardiac troponins are part of the actin/myosin/tropomyosin complex in cardiac muscle cells. There are three cardiac troponins, cardiac troponin T, cardiac troponin C and cardiac troponin I. In calcium-mediated cardiac muscle contraction, cardiac troponin T attaches the troponin complex to tropomyosin (Wells and Sleeper 2008). Cardiac troponin C serves as a binding site for calcium during excitation/contraction coupling and cardiac troponin I inhibits the interaction between actin-binding sites and myosin by inhibiting actomyosin ATPase (Wells and Sleeper 2008). When calcium binds with troponin C, troponin I is displaced and allows myosin to interact with actin (Wells and Sleeper 2008). When calcium is released from troponin C, troponin I moves back into a position which enables it to inhibit the interaction between actin and myosin again (Wells and Sleeper 2008). Troponins are present in the cytoplasm; therefore, an initial rise in cTnI can occur with mild cardiac damage when cytosolic troponin is released and may indicate reversible damage (Wells and Sleeper 2008; Serra, Papakonstantinou et al. 2010). A more marked and persistent increase is consistent with the release of structurally bound troponin proteins indicative of irreversible or ongoing myocardial damage (Wells and Sleeper 2008). Cardiac troponin I has been used to estimate severity of cardiac damage as well as estimate a prognosis following myocardial damage (Rajappa and Sharma 2005). In the case of acute myocardial infarction, the degree of increase of cTnI correlates with the risk of future cardiac problems or mortality (Wells and Sleeper 2008). The magnitude of increase of cTnI has also been used to predict the size of the myocardial infarct (Wells and Sleeper 2008). In dogs with cardiac disease, cTnI greater than 1.0 ng/ml or a persistent increase in cTnI are indicators of a poor prognosis (Fonfara, Loureiro et al. 2010). Children and adults with myopericarditis, however, can have markedly increased cTnI but these elevations are not associated with myocardial dysfunction or short term cardiac sequelae and therefore do not indicate a poor prognosis (Kobayashi, Aggarwal et al. 2012). Pediatric patients with chest trauma were also found to have increases in cTnI without significant myocardial dysfunction (Sangha, Pepelassis et al. 2012). Treatment in humans with increased cTnI is typically associated with acute

myocardial infarction (AMI) and centers around restoring perfusion; however, in other causes of increased cTnI the therapy is not defined or well understood (Twerenbold, Reichlina et al. 2011). It is important to recognize as well that significant heart disease can occur without increases in cardiac troponin I (Fennell and Forbes 2009).

Markers of Cardiac Damage in the Horse

The use of cardiac troponin I as a measure of myocardial damage in the horse has been investigated with certain disease states and toxicities as well as during exercise (Phillips, Giguiere et al. 2003; Begg, Hoffmann et al. 2006; Holbrook 2008; Lawler, Frye et al. 2008; Divers, Kraus et al. 2010; Durando, Birks et al. 2011; Nath, Anderson et al. 2012; Nath, Anderson et al. 2012; Slack, Boston et al. 2012). Cardiac troponin increases in 2-12 hours after myocardial injury, peaks in 18-24 hours following acute insult and stays increased for 7-10 days after the insult in people (Wells and Sleeper 2008). The half- life of cardiac troponin I in horses is reported to be 2 hours (Divers, Kraus et al. 2010). Human and equine cTnI are very similar and human assays have been used successfully in the horse (Rishniw and Simpson 2005); however, strict validation according to the American Society of Veterinary Clinical Pathology quality control principles (ACVP, 2009) for all assays has not been performed (Slack, Boston et al. 2012). The American Society of Veterinary Clinical Pathology requires that a test be validated by assessing linearity, precision, accuracy, analytical range, lower limit of detection and examining the effects of interfering substances (ACVP, 2009). Similar to findings in people with AMI, increased cTnI has been associated with severity of disease in horses with acute abdominal disease (Nath, Anderson et al. 2012). In horses with colic, an increased cardiac troponin I has been associated with myocardial dysfunction, ventricular arrhythmias and death (Diaz, Durando et al. 2009; Nath, Anderson et al. 2012; Radcliffe, Divers et al. 2012); however, it is not always accompanied by arrhythmias in horses with colic (Nath, Anderson et al. 2012). Horses that require colic surgery have a poorer prognosis if cardiac troponin I is increased at recovery (Radcliffe, Divers et al.

2012). Elevated cardiac troponin I has also been found to be associated with arrhythmias in horses with experimentally induced endotoxemia (Nostell, Brojer et al. 2012) and marked elevations in cardiac troponin I have been associated with ventricular tachyarrhythmias (Fennell and Forbes 2009). Also similar to humans, apparently healthy horses undergoing exercise can have elevations in cardiac troponin I (Holbrook, Birks et al. 2006). The significance of these elevations has not been determined (Holbrook, Birks et al. 2006; Slack, Boston et al. 2012). Although there are definitely associations between marked increases in cardiac troponin I and poor prognosis, horses with markedly elevated cardiac troponin I have recovered (Nath, Anderson et al. 2012) and serial cTnI measurements are a better indicator of prognosis than a single sample (Fonfara, Loureiro et al. 2010; Nath, Anderson et al. 2012).

Markers of Cardiac Damage Following Snake Envenomation

Cardiac troponin I has been used to diagnose myocardial damage following some pit viper envenomation (Lawler, Frye et al. 2008; Segev, Ohad et al. 2008; Pelander, Ljungvall et al. 2010); however, its use following rattlesnake envenomation is only sparsely documented (Lawler, Frye et al. 2008). In the presence of both skeletal muscle injury and cardiac injury such as occurs in many snake bite victims it is important to assess a biomarker that will differentiate skeletal from cardiac muscle damage. Since cardiac troponin I is not found in any other tissues it is a specific indicator of myocardial damage (Rajappa and Sharma 2005) and should be a useful marker to document myocardial damage following rattlesnake envenomation.

Electrocardiography – A General Overview

The electrocardiogram (ECG) is the most commonly used cardiovascular laboratory procedure and is the only practical method of recording the electrical activity of the heart (Fisch 1989). Most simply the electrocardiogram must consist of at least two electrodes, a positive and negative, which are connected to a recording instrument. Differences passed down the axis

between these electrodes will be recorded on the recording instrument and create what is recognized as an electrocardiogram (Holmes 1990). In the horse, systems with 4 electrodes are most common (Verheyen, Decloedt et al. 2010). One electrode serves as a reference and the other three each serve as part of a lead, activity measured between a negative and positive electrode (Verheyen, Decloedt et al. 2010). Although electrocardiograms can be used to give information regarding heart chamber size in humans and small animals, they can only be used to detect abnormal rhythms in the horse (Reed, Bayly et al. 2010). An ECG can be recorded at rest or during exercise. They can be measured for short or prolonged periods of time depending on the equipment available. Some arrhythmias may only be detected during exercise (Zucca, Ferrucci et al. 2003). Continuous ECG monitoring using telemetry or holter monitoring is necessary to detect infrequent arrhythmias and can be used to determine the severity of an arrhythmia (Desrochers 2011). Holter monitors are commonly used in human medicine and have been used in veterinary medicine as well (Raekallio 1992; Zucca, Ferrucci et al. 2003).

Most commonly the ECG is used in conjunction with other modes of detecting cardiac disease; however, sometimes an abnormal ECG is the only finding with a significant cardiac abnormality (Fisch 1989). Electrocardiogram and cTnI are the diagnostic cornerstones for diagnosing acute myocardial infarction in humans (Twerenbold, Reichlina et al. 2011). Clinically significant arrhythmias can occur in the absence of an increased cTnI (Pelander, Ljungvall et al. 2010). Electrocardiogram alone may be insufficient to diagnose AMI because there are other things that can cause similar ECG changes or because ECG abnormalities may not be present in people with AMI (Rajappa and Sharma 2005; Twerenbold, Reichlina et al. 2011). A normal ECG does not exclude cardiac disease (Reed, Bayly et al. 2010).

Electrocardiography Following Rattlesnake Envenomation

Electrocardiogram abnormalities are rarely reported in people following rattlesnake bite; however, they are well documented in dogs and horses (Schaer 1984; Dickinson, TraubDargatz et al. 1996; Rashmir-Raven and Brashier 2000; Willey and Schaer 2005; Lawler, Frye et al. 2008; Fielding, Pusterla et al. 2011). Arrhythmias in dogs are predominantly ventricular in origin although atrial fibrillation and premature atrial contractions have been reported (Schaer 1984; Willey and Schaer 2005). Arrhythmias that have been reported in rattlesnake bitten horses are third degree atrioventricular block (Dickinson, TraubDargatz et al. 1996; Lawler, Frye et al. 2008), supraventricular tachycardia (Dickinson, TraubDargatz et al. 1996), paroxysmal ventricular tachycardia with frequent ventricular premature complexes (Rashmir-Raven and Brashier 2000) and atrial fibrillation (Dickinson, TraubDargatz et al. 1996; Fielding, Pusterla et al. 2011).

Tumor Necrosis Factor alpha

Tumor necrosis factor alpha (TNF-α) is a cytokine which is predominantly released from macrophages but also glial cells in the brain, Kupffer cells in the liver, keratinocytes in skin, mast cells, T and B lymphocytes and natural killer cells when they are stimulated by an antigen such as lipopolysaccharide (Tracey and Cerami 1993). There is evidence that at very low concentrations TNF-α may regulate several physiological processes such as the circadian rhythm of body temperature, sleep and appetite; however, with increased systemic concentrations of TNF-α individuals can experience fever, loss of appetite, cachexia, and lethargy (Strieter, Kunkel et al. 1993). TNF-α is a pro-inflammatory agent that can induce macrophage differentiation, neutrophil degranulation and trigger the release of other inflammatory mediators such as IL-1, IL-6, IL-8 and the arachidonic acid metabolites (Moura da Silva, Laing et al. 1996). The primary protective role of TNF-α is to contain local infection (Murphy, Travers et al. 2008). Once TNF-α has been

released it activates the vascular endothelium resulting in an increase in vascular permeability. Plasma immunoglobulins and inflammatory cells are released into the tissues resulting in increased fluid drainage via the lymphatics. The stimulated endothelial cells express proteins that trigger blood clotting in the local small vessels resulting in obstruction of blood flow effectively containing the pathogen. In situations of overwhelming pathogen exposure, such as gram negative sepsis (Beutler and Grau 1993) or snake envenomation (Tu 1982), large amounts of TNF-α are produced not only locally but systemically by activated macrophages in the liver and spleen. This can result in profound vasodilation, reduction of blood pressure, increased vascular permeability, loss of plasma volume and ultimately shock (Murphy, Travers et al. 2008). TNF-α induced endothelial cell changes can have several effects. TNF-α stimulation of endothelial cells results in the release of factors all of which favor thrombosis such as tissue factor, platelet-activating factor and von Willebrand factor (Beutler and Grau 1993). TNF-α increases expression of cell adhesion molecules, particularly ICAM-1, resulting in increased adhesion of leukocytes as well as platelets further promoting coagulation (Beutler and Grau 1993). TNF-α also can cause the margination of neutrophils (Beutler and Grau 1993). TNF-α can stimulate the release of vasodilatory substances (prostaglandin E_2, prostacyclin I_2, thoromboxane A_2) and the potent vasoconstrictive substance endothelin (Beutler and Grau 1993).

The role of TNF-α has been examined in many different disease states in people, including snakebite (Strieter, Kunkel et al. 1993; Avila-Aguero, Paris et al. 2001; Mann 2001; Acikalin and Gokel 2012). TNF-α has also been investigated in the horse for a multitude of different conditions, not however, snake bite (Morris, Moore et al. 1991; Pusterla, Magdesian et al. 2006; Holbrook, McFarlane et al. 2010; Suagee, Burk et al. 2011; Suagee, Corl et al. 2012). The role of TNF-α specifically in heart disease has been examined (Mann 2001). TNF-α has been shown experimentally to have several effects on the heart which include left ventricular dysfunction, cardiomyopathy, abnormalities in myocardial metabolism, and cardiac myocyte

apoptosis (Mann 2001). Increased TNF-α concentration has been found in people with congestive heart failure and has been used as a marker of increased mortality in people who initially survive cardiopulmonary resuscitation (Strieter, Kunkel et al. 1993). Evidence that TNF-α is a major contributor in myocardial damage occurring secondary to pit viper envenomation was provided when mice given anti-TNF-α antibodies were resistant to the cardiotoxic effects of *Vipera aspi*s (Szold, Ben-Abraham et al. 2003). Viper venoms contain large amounts of zinc metalloproteinases that are very similar to endogenous matrix metalloproteinases (Moura da Silva, Laing et al. 1996). Venom metalloproteinases have been shown to cleave pro-tumor necrosis factor-α to the active form of TNF-α increasing the amount of the already present inflammatory cytokine (Moura da Silva, Laing et al. 1996). The increased concentration of TNF-α has been shown to contribute significantly to local tissue necrosis that occurs with pit viper envenomation (Moura da Silva, Laing et al. 1996). There is variation in the magnitude of increase in TNF-α following snake bite (Lomonte, Tarkowski et al. 1993; Petricevich, Teixeira et al. 2000). It is unknown if this variability is due to species of snake, route of administration of venom, or a combination of factors (Acikalin and Gokel 2012). The degree of TNF-α increase has been associated with clinical severity with higher levels of TNF-α being associated with a more severe clinical picture (Acikalin and Gokel 2012) in patients bitten by snakes in the genus *Vipera* (Acikalin and Gokel 2012). Some investigators have recommended using TNF-α concentration in snake bite victims to guide therapy (Acikalin and Gokel 2012).

Several treatments have been investigated to reduce the adverse effects of TNF-α in disease states. Monoclonal antibodies against TNF-α were evaluated in the treatment of gram negative sepsis and were very beneficial in humans (Beutler and Grau 1993). TNF-α inhibitors are now commercially available in the form of anti-TNF antibodies and TNF receptor fusion proteins but are not routinely used in veterinary medicine. TNF-α inhibitors are not without risk. A review of the human literature indicates that long term use of TNF-α inhibitors can lead to

serious side effects including serious bacterial, viral, and fungal infections, a possible increased risk for development of lymphoma, worsening of clinical signs and pathology in patients with congestive heart failure and others (Lin, Ziring et al. 2008). The adverse effects noted in patients with heart disease may make it an undesirable treatment if cardiac disease or damage is suspected; however, in the earlier stages of cardiac disease in a rat model of volume overload TNF-α inhibitors have been shown to attenuate adverse myocardial remodeling (Jobe, Melendez et al. 2009). Most adverse effects are reported following continued use for the control of chronic disease (Lin, Ziring et al. 2008).

Immune Response to Snake Venom

The immune response to several snake venoms has been well studied (Sadahiro, Kondo et al. 1978; Da Silva and Tambourgi 2011). Proteins, which make up approximately 98% of venoms, are the primary immunogens (Gutierrez, Leon et al. 2003). Many proteins in snake venoms are similar and antibodies made during an immune response to one snake venom may protect against different snake venoms. Cross reactivity between rattlesnakes has been shown (Ownby and Colberg 1990). Individual venom toxins' structure and molecular mass, amount of the toxin in the venom, dose of venom received, and several host factors affect the immune response to snake venom (Leon, Sanchez et al. 2011). Some toxins are more immunogenic than others and some have even been found to be immunosuppressive (Favoretto, Ricardi et al. 2011; Leon, Sanchez et al. 2011). When venom is injected into an individual, the initial response is predominantly inflammation dominated by neutrophils (Leon, Sanchez et al. 2011). Secondarily, the neutrophils are replaced by antigen presenting cells (predominantly macrophages with some dendritic cells) (Gutierrez, Leon et al. 2003). Venom toxins may immediately travel up lymphatics and come into contact with immunoglobulin receptors on resting B cells in the lymph nodes where they will activate B cells and induce secretion of IgM antibodies (Leon, Sanchez et al. 2011). Other venom toxins may be captured by antigen presenting cells in the local tissues,

travel to the lymph nodes and be processed and coupled with MHC II molecules for presentation on the cell surface (Leon, Sanchez et al. 2011). The toxins are presented to T helper cells. These T helper cells will be induced to transform into memory T cells and effector T cells. Activated T helper cells will interact with B lymphocytes which will be stimulated to differentiate into memory B cells and plasma cells that will be responsible for immunoglobulin production and differentiation (Leon, Sanchez et al. 2011). These memory B cells will play a role in the anamnestic immune response should there be another venom exposure. Humans have an anamnestic response to snake venom (Theakston, Reid et al. 1981; Pe, Aye-Aye-Myint et al. 1995). In people bitten by the king cobra, there is evidence that the humoral immune response to repeated envenomations is greater, more effective at neutralizing venom effects and longer lasting than that of a single envenomation (Pe, Aye-Aye-Myint et al. 1995). This has also been shown to be true in Waorani Indians bitten by poisonous snakes endemic to the area where they live (Theakston, Reid et al. 1981). Following natural envenomation, the persistence of circulating antibodies is highly variable in humans. In a human bitten by the puff adder (*Bitis arietans*) antibodies were measurable out to 81 days post envenomation (Theakston 1985). In two patients bitten by the king cobra (*Ophiophagus hannah*) titers lasted approximately 8 weeks (Pe, Aye-Aye-Myint et al. 1995). In goats experimentally envenomated with *Crotalus atrox* venom, antibodies were short lived (~60 days) (Glenn, Becker et al. 1970). Although it is uncertain how long venom antibody titers persist following natural envenomation, it has been shown that circulating anti-venom antibodies present at the time of or shortly after experimental envenomation are effective at decreasing the toxic effects of venom in mice (Rucavado and Lomonte 1996). People bitten multiple times often have more mild venom effects supporting the idea that vaccination may be helpful in preventing the adverse effects of snake envenomation.

Vaccination Against Snake Venom

Vaccinations are one of the most important contributions to public health in the past century (Murphy, Travers et al. 2008). Vaccinations were discovered by Edward Jenner when he realized that infection with a bovine form of small pox, called vaccinia, provided protection against human small pox. Vaccinia caused a brief and limited subcutaneous reaction and then provided long term immunity. Louis Pasteur decided to honor Edward Jenner's discovery by calling all subsequent discoveries of this type of immunity stimulated against pathogens as vaccination (Murphy, Travers et al. 2008). There are several common requirements for vaccines regardless of the immunogen. A vaccine must be safe and provide protection against the disease in a large number of vaccinates. It must generate antibodies as well as T cells directed against target epitopes and generate long lived immunity (Murphy, Travers et al. 2008). To accomplish the production of an effective vaccine one must know how the organism or substance causes disease. For example, is the organism intracellular or extracellular?

Production of vaccines against snake venom has been ongoing since at least 1887 (Sewall 1887). Initially, repeated sub-lethal venom injections were given to pigeons. These pigeons subsequently had milder responses to low doses of venom but still succumbed to higher doses of venom (Sewall 1887). In 1968 a group of people were immunized with detoxified venom of the Habu (*Trimeresurus flavoviridis*). Titers comparable to those reached when individuals were given anti-venom were not achieved; therefore, it was not considered successful (Leon, Sanchez et al. 2011). In 1978 proteinase-containing fractions of Habu were used to immunize monkeys (Sadahiro, Kondo et al. 1978). The monkeys were then challenged with crude venom and only those receiving the lowest dose of venom survived resulting in the conclusion that the vaccine was not going to be protective in a natural setting (Sadahiro, Kondo et al. 1978). Horses immunized with phospholipase A_2 were protected against the lethal effects of *Crotalus durissus*

terrificus venom (Dos-Santos, Yamaguchi et al. 1989) giving hope for the benefit of a venom vaccine.

The goal of a vaccine to be safe would require that a venom vaccine contain venom or venom components that would not make the animal systemically ill. Venom detoxification can occur by several mechanisms. Unfortunately, important epitopes may be lost during the detoxification process resulting in reduced immunogenicity (Leon, Sanchez et al. 2011). Epitopes may also be destroyed by venom proteinases if venom is not appropriately handled and processed prior to the vaccination process (Leon, Sanchez et al. 2011). These are considerations in the manufacture of anti-venoms as well as in vaccine development.

Robust and long lived immunity is another goal of a successful vaccine. Substances called adjuvants are coupled with antigens to improve the immune response. Several adjuvants have been used with snake venoms in the process of making anti-venoms. The most common adjuvants used are aluminum salts, incomplete Freund's adjuvant, and Freund's adjuvant (Leon, Sanchez et al. 2011). Freund's adjuvant results in the most robust immune response; however, historically, it has been associated with local tissue damage and granuloma formation (Leon, Sanchez et al. 2011). Most recently, Freund's adjuvant has been used in protocols which involve the injection of Freund's adjuvanted venom in small volumes in multiple anatomical sites to avoid the local tissue reactions previously seen yet still reap the benefits of the robust immune response stimulated with this adjuvant (Chotwiwatthanakun, Ronachai et al. 2001).

Snake Venom Detection – A General Overview

Snake venom has been detected in biological samples since 1902 (Lamb 1902). The first test was a precipitin test (Minton 1987). It was a simple test and was first used in post mortem samples from a person bitten by a cobra (Minton 1987). Subsequently, multiple different laboratory methods have been employed to detect snake venom since including bioassays,

immunodiffusion, immunoelectrophoresis, immunofluorescence, haemagglutination, radioimmunoassay (RIA), and enzyme-linked immunosorbent assay (ELISA) (Selvanayagam and Gopalakrishnakone 1999). Of these tests the radioimmunoassay is the most sensitive; however, it requires technical expertise and facilities involved in handling a radioisotope as well as expensive equipment; therefore, it is not suitable for field situations (Minton 1987). Detection of rattlesnake venom in biological fluids of veterinary patients could be beneficial not only as an aid for diagnosis in cases that aren't clinically apparent but also to direct therapy. The ideal assay to be used in veterinary medicine would be rapid, accurate and not cost prohibitive. It would have reagents that are stable, safe to handle, and simple to use. The ELISA is a much more likely candidate for use in field situations as it is relatively simple, reagents are inexpensive and stable, and it can be rapid (Minton 1987).

Snake Venom ELISA Development

ELISA technology has been used for the detection of North American pit viper venoms in experimentally envenomated animals (Minton, Weinstein et al. 1984) and rattlesnake venom in a human snake bite victim (Ownby, Reisbeck et al. 1996). Colorimetric assays using spectrophotometric detection of color change have been the standard in venom ELISAs. These assays involve the combination of a substrate with an enzyme labeled antibody to produce a color change. The most common enzyme label was originally horse radish peroxidase; however, alkaline phosphatase is now commonly used (Minton 1987). More recently, fluorometry has been used in ELISA development because of its superior sensitivity (Selvanayagam and Gopalakrishnakone 1999). Fluorometry involves combining, most commonly, alkaline phosphatase labeled antibodies with a fluorogenic substrate such as 4-methyl umbelliferyl phosphate (4 MUP) to produce fluorescence (Selvanayagam and Gopalakrishnakone 1999). One venom enzyme linked fluorometric assay detected venom levels up to 0.1 pg/ml (Bhatti, Wong et

al. 1993). It is believed, based on an empirical average dose of natural envenomation by North American rattlesnakes that an assay would need to detect 1 ug/ml of venom (Minton 1987).

Venom ELISAs involve binding anti-venom antibodies produced in animals to a plate and sandwiching with similar antibodies. If these antibodies are not purified for specific venoms or venom components high background can be an issue (Ho, Warrell et al. 1986). Non-specific reactivity can be a challenge with venom detection by ELISA. It can occur between the sandwich antibody and the plate or the coating antibody. In instances where venom is being detected in serum, high background may occur when all binding sites on the plate are not coated and other immunoglobulins in the serum react with the plate. Non-venom immunoglobulins in serum can also react with the coating or sandwiching antibodies (Ho, Warrell et al. 1986). Using one standard assay, 36% of normal healthy people tested positive for circulating venom (Voller, Bidwell et al. 1980). Using a different standard assay 63% of healthy individuals tested positive for 2 ng/ml of circulating venom which was the lower limit of sensitivity of the assay indicating false positive results (Ho, Warrell et al. 1986). Non-specific binding or background is likely to be the cause of a positive result in patients that have no signs of envenomation or the persistence of venom in individuals that have received adequate amounts of anti-venom and are clinically improving or normal (Lwin, Myint et al. 1984). These non-specific reactions can be decreased by adding a blocking antibody to bind to non-specific binding sites (Ho, Warrell et al. 1986). Care must be taken to choose a blocking antibody that will not react with the detecting antibody. For example, ruminant immunoglobulins such as ovine and bovine may cross react. Non-specific binding when detecting venom in human samples can also be reduced by using other biological samples, such as urine or a wound aspirate that are lower in protein than serum or plasma (Aleman, Nieto et al. 2005). Minton et. al. considered fluid aspirated from the bite site to be the ideal sample for their ELISA detecting North American Pit Vipers; however, positive wound aspirates don't necessarily confirm envenomation as venom could be present on the skin but not

have been injected through the skin (Ho, Warrell et al. 1986). Wound aspirates are also not a good sample to use when trying to quantify the amount of venom injected (Minton, Weinstein et al. 1984). Urine should be a better sample in this case. Venom has been quantified in a human snakebite victim's urine using ELISA (Ownby, Reisbeck et al. 1996). In the horse, urine has a high mucous content due to large numbers of goblet cells in the renal pelvis epithelium and compound tubular mucus glands (Savage 2008). This mucus secretion is thought to be a protective measure and a reason why horses do not commonly get cystitis (Savage 2008). This mucus is problematic when performing ELISA on horse urine because it can result in non-specific binding similar to extraneous proteins in serum or plasma (Ho, Warrell et al. 1986). Timing of sample collection becomes an issue when attempting to detect venom in urine as the excretion of venom following envenomation has not been specifically defined (Ho, Warrell et al. 1986). It has been suggested that venom can be present in the urine as soon as 30 minutes post envenomation and can be detected at least 56 hours after envenomation (Minton 1987). In a human snakebite victim venom was first detected in the urine at day 3 and persisted until day 5 at which time no more samples were analyzed (Ownby, Reisbeck et al. 1996). Another challenge with ELISAs is cross reactivity. This is a particularly common feature with rattlesnakes and is beneficial in the development of anti-venoms but detrimental in the development of species specific ELISAs (Minton, Weinstein et al. 1984). Although their venoms are unique in some ways, they have many common antigens (Minton, Weinstein et al. 1984). An ELISA developed for detection of North American pit vipers demonstrated wide cross reactivity across the species; however, reactions to homologous venom were always much stronger (Minton, Weinstein et al. 1984). This extensive cross reactivity among the North American pit vipers makes the use of ELISA for identifying the species of snake very difficult (Minton, Weinstein et al. 1984). Attempts have been made to isolate individual toxins that may be unique to a certain species of snake and use these for venom detection; however, cross reactivity has proven to still be a challenge (Weinstein, Minton et al. 1985; Lomonte, Moreno et al. 1987; Li and Ownby 1994).

Reagent quality affects ELISA results as well. When using antibody reagents typically polyclonal antibodies are used as they are the most readily available and inexpensive (Ho, Warrell et al. 1986). Theoretically monoclonal antibodies to specific venom components should have less cross reactivity; however, they may also be less avid than polyclonal antibodies and some do not adsorb well to polystyrene surfaces or handle the manipulations involved in enzyme labeling well (Ho, Warrell et al. 1986). Additionally, cross reactivity has been reported when using monoclonal antibodies (Pukrittayakamee, Esnouf et al. 1983) so the additional cost and time required for their production may not be justified.

With all of the opportunities for false positive results it is very important when developing a venom ELISA to have an adequate number of positive and negative control samples (Ho, Warrell et al. 1986). Importantly, the controls should be chosen within the same population as the snake bite patients that will be assayed (Ho, Warrell et al. 1986).

Minimal focus has been placed on development of assays that can differentiate between species of rattlesnakes because anti-venoms are polyvalent and there is a high level of cross reactivity. It has been more important in countries where anti-venoms are monospecific and little cross reactivity occurs (Ho, Warrell et al. 1986). Assays have shown the ability to differentiate between *Agkistrodon sp.* and *Crotalus sp.* which may be important as *Agkistrodon sp.* bite victims are much less likely to need anti-venom.

Hypotheses and Aims of the Study

While cardiotoxicity has been reported following rattlesnake evenomation in the horse (Dickinson, TraubDargatz et al. 1996; Lawler, Frye et al. 2008; Fielding, Pusterla et al.2011), the occurrence and severity are variable and there are no prospective studies examining the effects of rattlesnake venom on the horse's heart. We hypothesized that cardiotoxcity was common following rattlesnake envenomation. The cause of the cardiotoxicity that has been reported is

unknown. We hypothesized cardiotoxicity following rattlesnake envenomation in the horse could be a dose dependent effect where horses that receive more venom are more likely to experience cardiotoxic effects. Alternatively, we hypothesized that increases in TNF-α following rattlesnake envenomation could result in cardiotoxicity. We further hypothesized that horses that are bitten more than one time will develop anti-venom antibody titers which would be protective against the cardiotoxic effects seen following rattlesnake envenomation. Finally, we hypothesized that horses would develop anti-venom antibody titers in response to a rattlesnake toxoid vaccine that are similar to natural venom exposure. In order to test these hypotheses we developed the following specific aims:

-Develop an ELISA for the detection of rattlesnake venom in biological samples from clinically bitten horses as a means of estimating venom dosage.

-Measure cardiac damage in rattlesnake bitten horses by measuring cardiac troponin I (cTnI) and evaluating electrocardiograms for the presence of arrhythmias.

-Explore factors that contribute to cardiac damage following rattlesnake envenomation by comparing tumor necrosis factor alpha (TNF-α) and anti-venom antibody titers to markers of cardiotoxicity.

-Assess the potential for vaccination to prevent the clinical effects of rattlesnake envenomation comparing antibody titers in horses vaccinated with the rattlesnake venom toxoid to titers in horses bitten by rattlesnakes.

CHAPTER III

DEVELOPMENT OF A DOUBLE SANDWICH FLUORESCENT ELISA TO DETECT RATTLESNAKE VENOM IN BIOLOGICAL SAMPLES FROM HORSES WITH A CLINICAL DIAGNOSIS OF RATTLESNAKE BITE

Submitted for Publication

ELISA DEVELOPMENT BACKGROUND

Development of this assay began using a double sandwich colorimetric technique modified from Audebert et.al. (Audebert, Sorkine et al. 1992). Polystyrene plates were coated overnight at 4°C with 100 ul/well of 0.1 ug/ml Wyeth's Polyvalent (Crotalidae) Antivenin. Plates were washed five times with 200 ul/well of phosphate buffered saline (PBS) with 0.05% Tween-20 (pH 7.4) at this step as well as between each incubation. A volume of 200 ul/well of blocking solution composed of 5% donkey serum (Jackson ImmunoResearch Laboratories, Inc.) in PBS was added. This step and all subsequent steps the plates were incubated at room temperature for 1 hour. Samples were added at 200 ul/well. A sheep polyvalent crotalidae anti-venom (CroFab) was then added to each well (100 ul) at a dilution of 1:1000 in incubation buffer. Alkaline phosphatase labeled donkey anti-sheep IgG (Jackson ImmunoResearch Laboratories, Inc.) at 1:200 in Tris-buffered saline (pH 7.5) was added to each well at a volume of 200 ul/well. After the final washing step, the substrate p-nitrophenyl phosphate (PNPP) in 2-amino-2-methyl-1,3-propanediol (AMPD) buffer AMPD buffer was added (200 ul/well) and optical densities were measured at 15 minutes at 405 nm using an ELISA plate reader. The limit of detection of this

assay was 300 ng/ml and the goal for the assay was 1ng/ml. In an effort to reach greater sensitivity the assay was modified using fluorescence.

ABSTRACT

Rattlesnake bites in horses are not uncommon and the clinical outcomes are widely variable. Treatment of horses with anti-venom is often cost prohibitive and could have negative consequences therefore the development of a quantitative test to determine if anti-venom therapy is indicated would be valuable. The objective of this study was to develop an ELISA to detect rattlesnake venom in biological samples from clinically bitten horses. Nineteen horses were enrolled in the study. Urine was available from 19 horses and bite site samples were available from 9 horses. A double sandwich fluorescent ELISA was developed and venom was detected in 5 of 9 bite site samples and 13 of 19 urine samples. In order to determine if this assay is useful as a guide for treatment, a correlation between venom concentration and clinical outcome needs to be established. For this, first peak venom concentration needs to be determined. More frequent, consistent sample collection will be required to define a venom elimination pattern in horses and determine the ideal sample collection time to best estimate the maximum venom dose. This report describes development of an assay with the ability to detect rattlesnake venom in the urine and at the bite site of horses with a clinical diagnosis of rattlesnake bite.

INTRODUCTION

In certain geographical areas it is common for horses to be bitten by rattlesnakes. The clinical response of horses to rattlesnake venom appears to be highly variable (Dickinson, Traub-Dargatz et al. 1996; Rashmir-Raven and Brashier 2000; Lawler, Frye et al. 2008; Fielding, Pusterla et al. 2011; Gilliam, Holbrook et al. 2012). Horses bitten by rattlesnakes can experience cardiac damage evidenced by increased cardiac troponin I or abnormal electrocardiograms (Rashmir-Raven and Brashier 2000; Lawler, Frye et al. 2008; Gilliam, Holbrook et al. 2012).

These cardiac changes can lead to decreased performance, loss of use, or death (Dickinson, Traub-Dargatz et al. 1996; Rashmir-Raven and Brashier 2000; Lawler, Frye et al. 2008; Fielding, Pusterla et al. 2011; Gilliam, Holbrook et al. 2012). The reported mortality rates in horses are relatively high, varying from 9-25% compared to <1% in people (Dickinson, Traub-Dargatz et al. 1996; Weinstein, Dart et al. 2009; Fielding, Pusterla et al. 2011). The use of anti-venom is known to significantly reduce morbidity and mortality in people (Gold, Barish et al. 2004), however treating an adult horse with anti-venom is often cost prohibitive. Additionally, the only anti-venom products available on the veterinary market are equine origin products. Serum sickness has been reported in horses secondary to the administration of equine origin biologics (Guglick, MacAllister et al. 1995; Smith 1996; Aleman, Nieto et al. 2005). We hypothesized that the severity of clinical signs seen with rattlesnake envenomation is directly related to the quantity of venom received and that knowing the amount of venom that a horse received would be beneficial in determining whether or not anti-venom is necessary as well as provide helpful information when giving horse owners a prognosis. Detection of rattlesnake venom has not been reported in equine biological samples, however an ELISA that identifies Australian snake venom (Forbes and Church 2010) is widely used for venom detection in horse urine as well as other species because of its low cost, high sensitivity, and rapidity (Hung, Liau et al. 2003). We developed a double sandwich fluorescent ELISA to detect venom at the bite site as well as in the urine of rattlesnake bitten horses.

MATERIALS AND METHODS

Materials

Crotalus viridis viridis lyophilized venom generously donated from the venom laboratory at Oklahoma State University was used in the development of this assay. Anti-venoms used were

equine polyvalent crotalidae anti-venom[1] and ovine polyvalent crotalidae anti-venom[2]. Ninety six well flat bottom microtiter ELISA plates[3] were purchased from Thermo Scientific. Lyophilized donkey serum (60mg/ml)[4] and alkaline phosphatase labeled donkey anti-sheep IgG (0.6 mg/ml)[5] were purchased from Jackson ImmunoResearch Laboratories, Inc. 4-methylumbelliferyl phosphate (0.6mM)[6] was purchased from Sigma.

ELISA for rattlesnake venom

Ninety-six well, flat bottom polystyrene microtiter plates were coated using 50µl per well of sodium bicarbonate coating buffer (pH 9.6) containing 0.1 µg/ml horse polyvalent crotalidae antivenom[1] and incubated at room temperature overnight. Plates were washed three times using PBS (pH 7.4), then blocked using 200µl 5% donkey serum per well and incubated at room temperature overnight. Plates were washed three times using PBS, then donkey serum was added again (200µl 5% donkey serum per well) and plates were incubated for 1 hour at room temperature at which time the wash and donkey serum incubation steps were repeated. Samples were centrifuged at 2000 RPMs for 15 minutes and then urine and bite site samples were diluted 1: 5 and 1:10 respectively with incubation buffer (PBS/.05% Tween-20). Control urine and wound samples were handled the same as clinical samples. All samples were then added in triplicate (50µl/well) and incubated for 1 hour at room temperature. Plates were washed three times using PBS. Incubation buffer containing 10 µg/ml sheep polyvalent crotalidae anti-venom was added (100µl/well) and the plate was incubated at room temperature for 1 hour. Plates were washed five times using PBS. Tris Buffered Saline (pH 7.4)[7] containing alkaline phosphatase labeled donkey anti-sheep immunoglobulin was added (50 µl/well) and the plate was incubated at

[1] Antivenin (Crotalidae) Polyvalent, Fort Dodge, Overland Park, KS
[2] Cro Tab generously donated by Dr. Steve Mackessy
[3] Immulon 4-HBX, Thermo Scientific, Pittsburg, PA
[4] Donkey serum, Jackson Immunolabs, Westgrove, PA
[5] Alkaline Phosphatase-conjugated Affinipure donkey anti-sheep IgG, Jackson Immunolabs, Westgrove, PA
[6] 4-methylumbelliferyl phosphate liquid substrate system, Sigma, St. Louis, MO
[7] Tris Buffered Saline, Fisher Scientific, Waltham, MA

room temperature for 1 hour. A conjugate dilution of 1:200 was used for bite site samples, while a 1:50 dilution was used for urine samples. The plate was washed using PBS-Tween six times with one minute soaks in between each wash. A 1:5 dilution of 4-methylumbelliferyl phosphate (6mM) with deionized water was added at 200µl/well under a hood and the plate was incubated in the dark for 15 minutes. Plates were read immediately using a spectrophotometer[8] at an excitation of 355nm and emission of 460nm. Venom concentrations were calculated based on the standard curve. A standard curve was run on each plate. Each sample was assayed in triplicate.

Biological Samples

Nineteen horses that were diagnosed with rattlesnake bite based on history and clinical signs were sampled. If the bite site was visible a cotton tipped swab was used to collect fluid from the bite wound at presentation. The swab was immediately placed in 1ml of 0.9% sterile saline and frozen in a liquid nitrogen tank until shipping. Urine was collected from horses at presentation, 24, 48, 72, and 96 hours, 1 week and 1 month post presentation. Urine was separated into 5ml aliquots and frozen in a liquid nitrogen tank until shipping. When all samples were collected they were shipped on dry ice to Oklahoma State University and stored at -80°C until assayed.

Wound swabs were taken from ten horses that had wounds that were not associated with rattlesnake bite. These swabs were handled and stored similarly and were used as negative controls. Urine was collected from normal healthy horses and was collected and handled similarly to the clinical samples to serve as a negative control.

[8] SpectromaxM2 Microplate Reader, Molecular Devices, Sunnyvale, CA

RESULTS

Bite site ELISA

Figure 1 is a representative standard curve from a plate measuring rattlesnake venom in the bite site samples. There was a strong correlation (r^2=0.98) between expected and observed concentration of venom at concentrations between 1ng/ml to 1 mg/ml. Bite site swabs were considered positive if they were greater than two standard deviations above the negative control swabs. Venom was detected in 5 of 9 bite site swabs (Figure 2).

Urine ELISA

Figure 3 is a representative standard curve from a plate measuring rattlesnake venom in urine samples. There was a strong correlation (r^2= 0.99) between expected and observed concentration of venom at concentrations between 100 ng/ml to 100 ug/ml. Urine samples were considered positive if they were greater than two standard deviations above the negative control urine sample. Venom was detected in urine samples from 13 of 19 horses at one or more time points. Figure 4 shows peak venom concentrations detected. Venom was detected in the 96 hour post presentation urine sample in 53.8% of horses which was the largest number of horses at any given time point; however, this finding was not statistically significant. Venom was detected in urine samples at all time points including the one month sample in 3 horses (Table 3). There was no correlation between the amount of venom detected at the bite site and the amount of venom detected in the urine.

Figure. 1. Standard curve of *Crotalus viridis viridis* in incubation buffer for bite site and control wound swab samples. Using the fluorescent sandwich-ELISA method, excitation 355, emission 455. Each concentration was assayed in triplicate and recorded as mean ± SD.

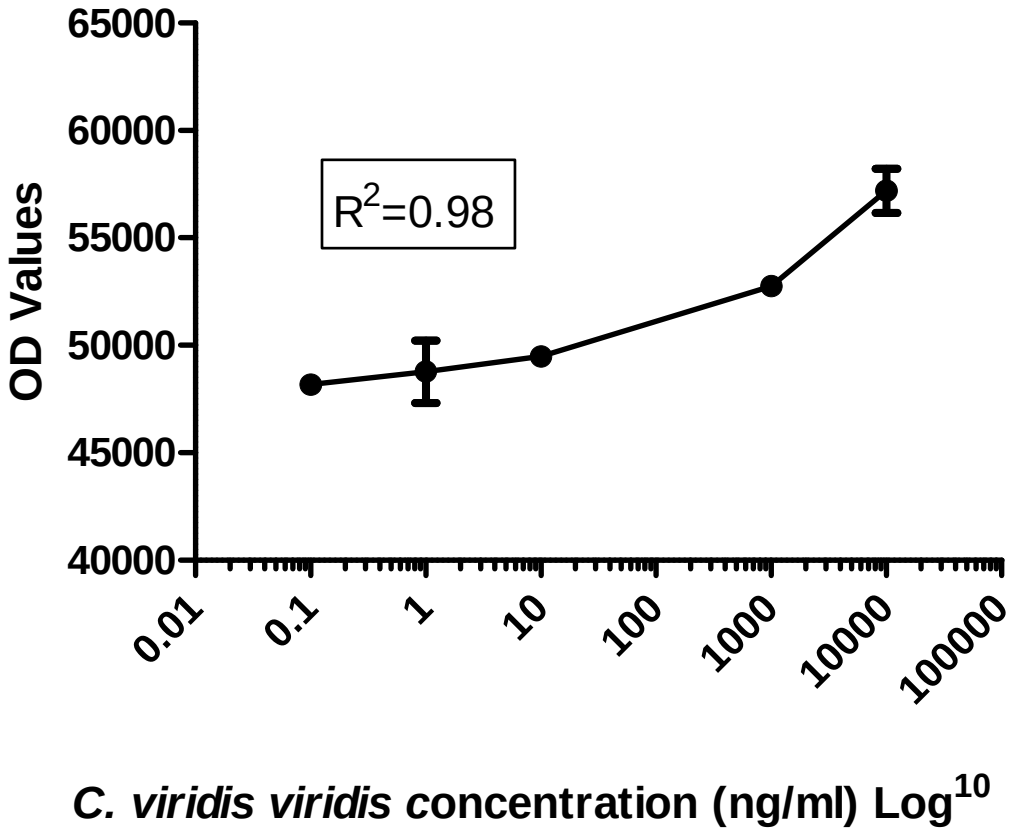

Figure. 2. Bite site and wound swab venom concentrations. Samples containing venom concentrations that were greater than 2 SD above the control were considered positive denoted with an asterisk.

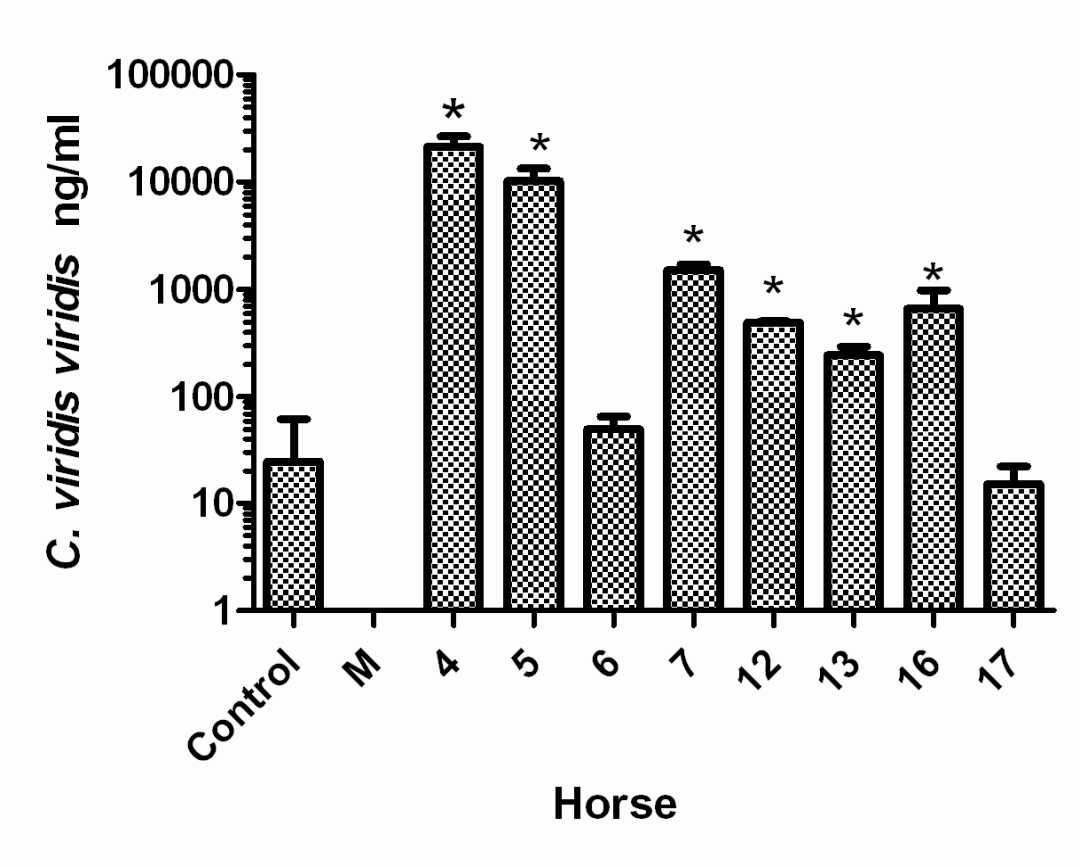

Figure. 3. Standard curve of *Crotalus viridis viridis* in normal healthy horse urine. Using the fluorescent sandwich-ELISA method, excitation 355, emission 455. Each concentration was assayed in triplicate and recorded as mean ± SD.

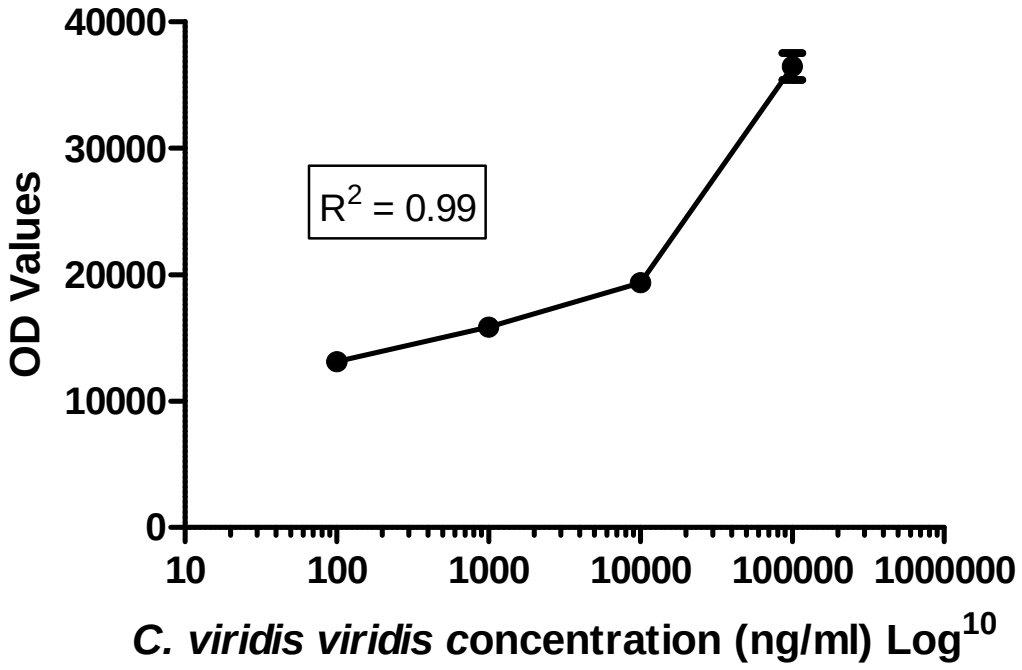

Table 3. Time point of venom positive urine samples. This table shows the number of horses that had positive urine samples at each given time point. Notice not all horses were sampled at each time point.

Sample Collection Time	**Number of horses positive/Number horses collected *(%)***
Presentation	7/15 *(46.7%)*
24 hr	6/13 *(46.2%)*
48 hr	5/13 *(38.5%)*
72 hr	5/12 *(41.7%)*
96 hr	7/13 *(53.8%)*
1 wk	3/11 *(27.3%)*
1 mo	3/11 *(27.3%)*

DISCUSSION

There are no previous reports of venom detection in the urine or at the bite site of horses bitten by North American rattlesnakes. Consequences of rattlesnake bite in the horse range from local tissue swelling and necrosis to cardiac damage, congestive heart failure and death. Currently there is no quantitative method for determining which horses may experience more severe effects after envenomation. Predicting which horses are most likely to experience more severe complications from rattlesnake bite would allow for the judicious use of anti-venom. In humans bitten by cobras determining the amount of venom in circulation was beneficial in guiding the use of anti-venom (Hung, Liau et al. 2003). Anti-venom is most beneficial when given early post-envenomation (Chippaux and Goyffon 1998); therefore in order for a test to be beneficial in guiding the use of anti-venom, it must be readily available. The ultimate goal of this research is to develop an ELISA that could be used stall side; therefore sample processing protocols were chosen to be practical in a clinical setting. Bite site samples followed by urine have been the preferred samples for venom identification in other snake venom assays (Forbes and Church 2010). In cats, cobra venom was detected most reliably in plasma up to 8 hours after envenomation, but venom was detectable in urine for at least 24 hours at concentrations more than forty times greater than those found in plasma (Moisidis, James et al. 1996). Although Australian snake venom has been successfully detected in the urine of horses and cats (Moisidis, James et al. 1996; Forbes and Church 2010) and rattlesnake venom has been detected in the urine of people (Ownby, Reisbeck et al. 1996), the excretion and metabolism of rattlesnake venom in the horse has not been well defined.

Horse urine has high mucous content as well as large amounts of calcium carbonate crystals (Reed, Bayly et al. 2010) which could interfere with the detection of venom using ELISA technique; however, detection of Australian snake venom in horse urine using a commercial kit has been successful and these authors concluded that mucous and calcium carbonate crystals did

not interfere with ELISA results (Forbes and Church 2010). Urine samples assayed using this commercial venom detection kit were diluted 1:1 (Forbes and Church 2010). In contrast, high background was a constant challenge in the development of the fluorescent ELISA reported here. In order to decrease background the urine was centrifuged and then diluted. Other processing methods such as ultra filtration and affinity chromatography were considered; however, adequate sensitivity with minimal processing was considered a necessity in order for this to result in a clinical test. The use of polyvalent antibodies could also contribute to higher background (Li and Ownby 1994). Commercial polyvalent anti-venoms were used in an effort to develop an assay with reagents that were readily available. Centrifugation plus dilution were found to be adequate to decrease background to an acceptable level in order to detect venom in the clinical biological samples.

Bite site swabs were collected from nine horses. These samples would be most useful in determining whether or not a horse had been bitten but were not likely to be useful in determining the venom dose. In order to determine the potential for interfering substances in wound exudates to cause false positive test results, swabs were taken from wounds not associated with rattlesnake bite and used as negative controls. Background values were found to be increased with non-rattlesnake bite associated wounds when compared with incubation buffer alone, so the bite site and control wound samples were centrifuged and diluted as well.

When working with crude venom and polyvalent anti-venoms it can be difficult to achieve a tight standard deviation. In order to decrease the likelihood of a false positive result during assay development we did not consider a sample positive unless it was greater than two standard deviations above the negative control. Considering a sample positive if it was greater than one standard deviation may be acceptable in a clinical setting where a false negative could result in withholding more aggressive treatment and therefore be more detrimental than a false positive.

Nine horses had both urine and bite site samples available for assay. Of these nine horses only one did not have venom detected in either the bite site sample or the urine. All other horses had venom detected in one or both samples. Venom was not detected in the bite site sample of four horses. Three of these horses had venom detected in the urine. There are several possible reasons for a negative bite site swab yet a positive urine sample. The time to presentation may play a key role in whether or not venom is detected at the bite site, however in horses the time of bite is often unknown. Venom on the skin may become desiccated and be undetectable. Swelling in the area of the wound may prevent exact identification of the bite site; therefore the area may be missed when the swab is taken. If venom was injected deeper into the tissues, venom may not remain on the skin; however, it would be in systemic circulation and therefore if in high enough concentrations should eventually be detectable in the urine. Inability to detect venom in either the bite site or the urine is likely indicative of a dry bite. One horse had venom detected at the bite site but did not have venom detected in the urine. It is most likely that very little if any venom entered systemic circulation. Perhaps the bite was very superficial and venom was readily available around the bite site but did not penetrate deeper into the tissues. There was no correlation between the amounts of venom detected at the bite site versus the amount detected in the urine. Detecting a large amount of venom at the bite site did not equate to detecting large amounts of venom in the urine.

Venom clearance may be widely variable and dependent on several factors such as amount of swelling at the bite site, perfusion of the envenomated tissues, dehydration and renal perfusion, individual animal immunologic response to envenomation, venom distribution in the tissues and likely others. These samples were collected from clinical cases; therefore certain factors could not be controlled. One major factor was the time to presentation after the bite. It would be ideal to have urine samples beginning a minimum of 2 hours post envenomation and every 12 hours until venom is no longer detected in order to better predict when peak elimination

of venom is most likely to occur (Minton, 1987). This was not possible in our clinical samples. Due to all of our patients being housed outdoors and examined at most twice daily, the closest estimation to time of bite was within a 12 hour window. Urine samples were planned to be collected every 24 hours from presentation until 96 hours post presentation, however, not all samples were available on all horses. Because of these sample timing discrepancies we were unable to accurately estimate peak venom concentration. One interesting note was the presence of venom in the urine of three horses in the one month sample. Viperid snake venoms consist largely of high molecular mass toxins that are characterized by a rapid initial absorption into local tissues followed by a slower absorption phase out of local tissues into systemic circulation and other tissues (Gutierrez, Leon et al. 2003). The delay in venom leaving the tissues results in delayed excretion of these venom components. Reports of coagulopathy up to 14 days post envenomation suggest that coagulopathic venom toxins remain in the body for a prolonged period of time (Seifert and Boyer 2001). To the author's knowledge there are no other published reports of venom detected in biological samples as late as 30 days post envenomation.

Our patients originated from the Texas panhandle, and central, western and eastern Oklahoma. Snakes endemic to these areas are *Crotalus viridis viridis, Crotalus atrox, Crotalus horridus horridus, Sistrurus miliarius streckeri, Agkistrodon contortrix laticinctus,* and *Agkistrodon piscivorus leucostoma.* Anti-venoms that were readily commercially available were used in our study and contained antibodies to *Crotalus adamanteus, Crotalus atrox, Crotalus scutulatus scutulatus, Agkistrodon piscivorus, Crotalus durissus terrificus,* and *Bothrops atrox*. Cross reactivity does occur amongst rattlesnake species (Minton, Weinstein et al. 1984; Perez, Garcia et al. 1984; Minton 1987; Ownby and Colberg 1987; Berger and Bhatti 1989); therefore, although antibodies to all species endemic to the study area were not included in the polyvalent anti-venoms used in this ELISA, detection should still occur. Cross reactivity has been demonstrated between *Crotalus viridis viridis, Crotalus horridus horridus, Crotalus atrox* and

Crotalus adamanteus (Minton 1987; Ownby and Colberg 1990; Li and Ownby 1992). The use of polyvalent anti-venoms in this assay makes determining the species of snake impossible. One of the horses in this study came from an area where *Agkistrodon piscivorus* is endemic. One of the antivenoms[2] used in this ELISA contains antibodies to *Agkistrodon piscivorus,* the second anti-venom[1] in the sandwich ELISA does not. Cross reactivity has been reported between Agkistrodotoxin and Crotoxin and Phospholipases A_2 contained in the venoms of a snake in the *Agkistrodon* genus and *Crotalus* genus respectively (Choumet, Ming-Shi et al. 1991). Further testing would be required to rule out cross reactivity with *Agkistrodon piscivorus* in this horse. The use of monovalent antibodies made against specific venom components is necessary to be able to differentiate between species of snake (Li and Ownby 1994). Identifying toxins specific to only one type of rattlesnake could prove difficult due to the many similarities in rattlesnake venoms (Martinez, Huang et al. 1989). Modalities used to accomplish this would likely be cost prohibitive and not rapid enough to be appropriate in a clinical setting.

RELEVANT FINDINGS

The ELISA was successful in detecting rattlesnake venom in equine biological samples. The purpose of developing this assay was to be able to quantify venom in clinical samples and determine if the amount of venom that a horse receives when bitten correlates with their clinical signs, in particular cardiac damage.

CHAPTER IV

CARDIOTOXICITY, INFLAMMATION, AND IMMUNE RESPONSE AFTER RATTLESNAKE ENVENOMATION IN THE HORSE

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ABSTRACT

Cardiac abnormalities are reported in rattlesnake bitten horses. The prevalence and cause are unknown. The objectives of this study were to detect cardiac damage in rattlesnake bitten horses by measuring cardiac troponin I (cTnI) and evaluating ECG recordings for presence of arrhythmias and to explore causes of this cardiac damage by measuring venom excretion, anti-venom antibodies, and tumor necrosis factor alpha (TNF-α).

In a prospective clinical study, bite site swabs, blood samples and urine samples were collected at various time points from 20 horses with a clinical diagnosis of snake bite. Continuous ECG recordings were obtained on the 20 affected horses and 6 normal control horses using 24-hour holter monitors. Plasma samples were assayed for cTnI, serum samples were assayed for TNF-α and anti-venom antibodies, and bite site swabs and urine were assayed for venom.

Forty percent of rattlesnake bitten horses (8/20) experienced myocardial damage (increased cTnI). Seventy percent (14/20) experienced a cardiac arrhythmia. There was a

positive correlation between cTnI and TNF-α ($p<0.02$). Horses with cTnI $\geq$2 ng/ml were more likely to have antibody titers >5000 ($p<0.05$). No correlations were found between venom concentration and cTnI, anti-venom antibody titers, TNF-α, or presence of arrhythmias.

INTRODUCTION

Rattlesnake envenomation in the horse, as well as other species, can have devastating outcomes. The mortality rate reported in horses after rattlesnake envenomation varies from 9% (Fielding, Pusterla et al.) to 25% (Dickinson, Traub-Dargatz et al. 1996), considerably higher than mortality rates reported in dogs (1%) (Hackett 2002) and people (<1%) (Weinstein 2009). The difference in these mortality rates is unknown and could be due to differences in treatments among species or could indicate a difference in sensitivity to venom among species. The most common manifestations of rattlesnake envenomation in horses are mild to severe swelling around the bite site, tissue necrosis and coagulopathy (Fielding, Pusterla et al.; Dickinson, Traub-Dargatz et al. 1996). Although cardiac abnormalities are not a common feature after snake bites (Vincent 2002), they have been reported in horses and can be important contributors to morbidity, future athletic potential or mortality (Dickinson, Traub-Dargatz et al. 1996; Rashmir-Raven and Brashier 2000; Lawler, Frye et al. 2008). Cardiac abnormalities also have been reported in dogs (Willey and Schaer 2005) and new world camelids (Dykgraaf, Pusterla et al. 2006) after rattlesnake bite. Venom is a complex mixture of proteins and, although some snake venoms contain toxins that exert specific effects on the heart (Zhang, Lader et al.; Harvey 1991), the exact cause of cardiac toxicity after rattlesnake envenomation in the horse is unknown. There are no prospective studies evaluating cardiac abnormalities after rattlesnake envenomation in the horse, and the cause of this damage has not been investigated.

Myocardial damage in the horse may be detected by recording an ECG or measuring cardiac troponin I (cTnI). Cardiac troponin I is a sensitive tool for documenting myocardial

damage in the horse (Divers, Kraus et al.), whereas electrocardiography is an insensitive indicator of myocardial damage (Fonfara, Loureiro et al.; Tacker, Van Vleet et al. 1979; Ettinger and Feldman 2005). Focal insult to the electrical system of the heart can result in arrhythmias in the absence of increases in cTnI (Pelander, Ljungvall et al.). We hypothesized that horses bitten by rattlesnakes frequently experience cardiac damage which may or may not be detected upon physical examination. In order to detect both myocardial cell injury and electrical dysfunction, cTnI was measured and 24-hour continuous ECG was recorded.

Rattlesnake envenomation in horses commonly incites a profound local and systemic inflammatory response (Smith 2009). It is unknown whether cardiac damage after rattlesnake bite is a consequence of the systemic inflammatory response, if it is caused by components of the venom itself or a combination of both. Tumor necrosis factor alpha (TNF-α) plays a role in the inflammatory response to rattlesnake envenomation (Moura da Silva, Laing et al. 1996). In addition to the body's own metalloproteinases, venom metalloproteinases process pro-TNF-α to its active form resulting in a marked increase in circulating TNF-α (Moura da Silva, Laing et al. 1996). In rats, TNF-α has been implicated in cardiac damage after pit viper envenomation (Szold, Ben-Abraham et al. 2003). TNF-α has not been measured in horses bitten by rattlesnakes. We hypothesized that the degree of cardiac damage may be associated with increases in circulating TNF-α concentration or venom concentration or both.

Considering that all study horses lived in an area endemic to rattlesnakes, previous exposure to rattlesnake venom was possible. Ruling out previous exposure based on history was considered unlikely due to multiple previous owners of individual horses. We further hypothesized that pre-existing anti-venom antibodies would be protective against the cardiotoxic effects of rattlesnake envenomation (Dos-Santos, Yamaquchi et al. 1989).

MATERIALS AND METHODS

Horses with a clinical diagnosis of snake bite defined as the presence of a characteristic snakebite wound, acute, severe focal swelling, and an owner's witness of a snake bite or both were enrolled in this prospective clinical study. Each horse received a complete physical examination. Treatment of all horses was at the discretion of the attending veterinarian. Horses were maintained in the hospital for a minimum of 1 week for sample collection and returned at 1 month after presentation for 24 hours. If the bite site was visible, cotton swab samples of the site were placed in 1 ml of sterile saline, immersed in liquid nitrogen, and stored at -80C until assays were performed. Thirty-four milliliters of whole blood was collected from the jugular vein of each horse at presentation, 24, 48, 72, 96 hours, 1 week and 1 month after presentation for serum and plasma samples. Serum and heparinized plasma aliquots were immersed in liquid nitrogen and stored at -80C until assays were performed. Urine samples were collected at each blood sampling time point. If horses did not urinate, they were sedated using xylazine (0.11 mg/kg), acepromazine (0.33 mg/kg), or a combination and urine was collected by urinary catheterization. Urine aliquots were immersed in liquid nitrogen and stored at -80C until assays were performed. A complete sample set was not obtained on every horse in the study. The study protocol was approved by the Institutional Animal Care and Use Committee at Oklahoma State University.

Cardiac Troponin I

Plasma samples were analyzed for cTnI using a 2-site sandwich colorimetric assay by use of a fluorometric analyzer[9] with an analytical sensitivity of 0.03 ng/ml. The range of detection of the assay is 0-50 ng/ml. This assay has been validated for use in horses and has been used in previous studies of horses (Durando 2006; Holbrook 2006). Cardiac troponin I remains stable in

[9] Stratus® CS STAT Fluorometric Analyzer, Dade Behring Inc., Newark, NJ

serum samples stored at room temperature for 5 days and was not affected by up to 5 freeze-thaw cycles (Wells 2008).

Holter monitor recording

Rattlesnake bitten horses were fitted with a Holter monitor[10] that recorded 24-hour continuous ECG at presentation, 1 week and 1 month after presentation. Normal horses were fitted with a Holter monitor for one 24-hour period. Recordings were downloaded using Holter software[11], randomized and interpreted by a blinded board-certified cardiologist who indicated the presence or absence of arrhythmia and defined the types of arrhythmias present.

Venom Analysis

Urine samples and bite site swab samples were assayed for venom using standard flurometric double sandwich ELISA technique. Horse anti-venom antibodies[12] and sheep anti-venom antibodies[13] were used as sandwich antibodies and alkaline phosphatase-labeled donkey anti-sheep antibodies[14] were used as detection antibodies. The substrate was 4-methyumbelliferyl phosphate (4-MUP)[15]. Plates[16] were read at 355 excitation/ 460 emission using a spectrophotometer[17]. A standard curve was constructed for each plate and the limit of detection was $\geq$ 2 standard deviations above the negative control.

[10] Digitrak-Plus 24Hr, Philips Medical Systems, Andover, MA

[11] Zymed Holter 2010 for Windows®, Philips Medical Systems, Andover, MA

[12] Anti venin (Crotalidae) Polyvalent, Fort Dodge Laboratories, Inc., Fort Dodge, IA

[13] Cro-Tab, University of Northern Colorado, Greeley, CO.

[14] Donkey anti-sheep IgG alkaline phosphatase labeled, Jackson ImmunoResearch. West Grove, PA.

[15] 4-methylumbelliferyl phosphate, Sigma-Aldrich, St. Louis, MO.

[16] Immulon HB4X, Thermo Fisher Scientific Inc.,

[17] SpectromaxM2 Microplate Reader, Molecular Devices, Sunnyvale, CA.

Tumor necrosis factor alpha

TNF-α was measured using a commercial equine specific ELISA[18] (McFarlane 2008). Plasma samples were thawed, diluted 1:4 using 4% BSA in Dulbecco's PBS (pH 7.4) and incubated at 37C for 1 hour before measurement.

Anti-venom antibodies

Serum samples taken at presentation and 1 week or 1 month or both were assayed for antibodies against *Crotalus atrox* using standard ELISA techniques. Anti-equine alkaline phosphatase antibody[19] was used as a detection antibody. Plates were read at 405 nm on a spectrophotometer[20] and titers were calculated based on the standard curve control. Titers $\geq$200 were considered positive.

STATISTICAL METHODS

Cardiac troponin I in bitten horses was compared with the normal range for horses established by the laboratory performing the assay. An exact binomial test was used to determine if bitten horses were more likely to have an increased cTnI then the normal population. A Fisher's Exact Test was used to evaluate the presence of arrhythmias in normal versus bitten horses as well as bitten horses with and without increased cTnI. Fisher's Exact Test also was used to evaluate the relationship of cTnI (increased/not increased above 2 ng/ml) with the presence of antibody titers > 5000. A Spearman's rank correlation coefficient was used to identify correlations between cTnI, TNF-α, venom, and anti-venom antibody titer.

[18] Equine TNF alpha Screening Set, Endogen, Pierce, Rockford, IL.

[19] Anti-equine alkaline phosphatase antibody, Sigma, St.Louis, MO

[20] THERMOmax microplate reader, Molecular Devices, Sunnyvale, CA

RESULTS

Twenty horses were included in the study. Horses resided in the Texas panhandle (12), west Texas (4), southeastern Oklahoma (2), north central Oklahoma (1), and western Oklahoma (1). Fourteen horses were bitten on the muzzle, 2 on the distal limb, 1 on the head midway between the right ear and eye, and bite location was not recorded in 3 horses. Treatments were recorded in 15 horses and included corticosteroids (13/15), non-steroidal anti-inflammatory drugs (14/15), antibiotics (13/15), and tetanus toxoid (2/15). Long-term follow-up was possible on 15 of the 20 horses. All 15 were alive at 1 month but 1 horse died acutely at pasture 48 days after initial presentation. Other clinical signs reported included diarrhea (1 horse) and severe myonecrosis at the site of an IM injection (1 horse).

Forty percent of the horses had increased cTnI (8/20) (range, 0.08 – 59.58 ng/ml; reference range, 0.00-0.06 ng/ml). Seventy percent (14/20) of the bitten horses had an arrhythmia confirmed on at least 1 time point and 2 of 6 normal horses had arrhythmias ($p=0.16$) (Table 4). Three horses that had arrhythmias at presentation still had arrhythmias at 1 month. One horse only had an arrhythmia at the 1 month evaluation. All horses with increased cTnI (8) also had arrhythmias. Six horses had arrhythmias without increased cTnI, but horses with increased cTnI were more likely to have arrhythmias than horses with normal cTnI ($p<0.04$). All 3 horses with arrhythmias present both at presentation and 1 month had increased cTnI concentrations.

TNF-α was highly variable among horses. There was a positive correlation between TNF-α concentrations and cTnI ($p<0.02$).

Titers were measured at presentation and 1 week or one month or both in 15 horses. None of these horses had titers above assay background at presentation. Thirteen of 15 horses

had positive antibody titers (>200). Manufacturers of a commercial rattlesnake toxoid vaccine[21] consider titers > 800 to be protective against the lethal effects of rattlesnake venom. Eleven of 15 horses had titers >800. When titers were < 5000 there was no positive correlation between antibody titer and cTnI ($p>0.05$), but horses with a cTnI >2 ng/ml were more likely to have markedly increased titers (> 5000) ($P<0.04$) at 1 week or 1 month. Not all horses had samples available at 1 week and 1 month, but when both samples were available, the highest titer was used in statistical analysis.

Venom was detected in 5 of 9 bite site swabs and in urine of 13 of 19 horses on at least 1 time point. Venom excretion in the urine was variable and tended to occur on or after day 3 of presentation. The earliest time point post-bite that venom was detected was within the first 24 hours, and venom was detected in the highest number of horses (7/13) at 4 days. There was no correlation between urine venom concentration and cTnI ($p>0.05$), TNF-α ($p>0.05$), or anti-venom antibody titers ($p>0.05$).

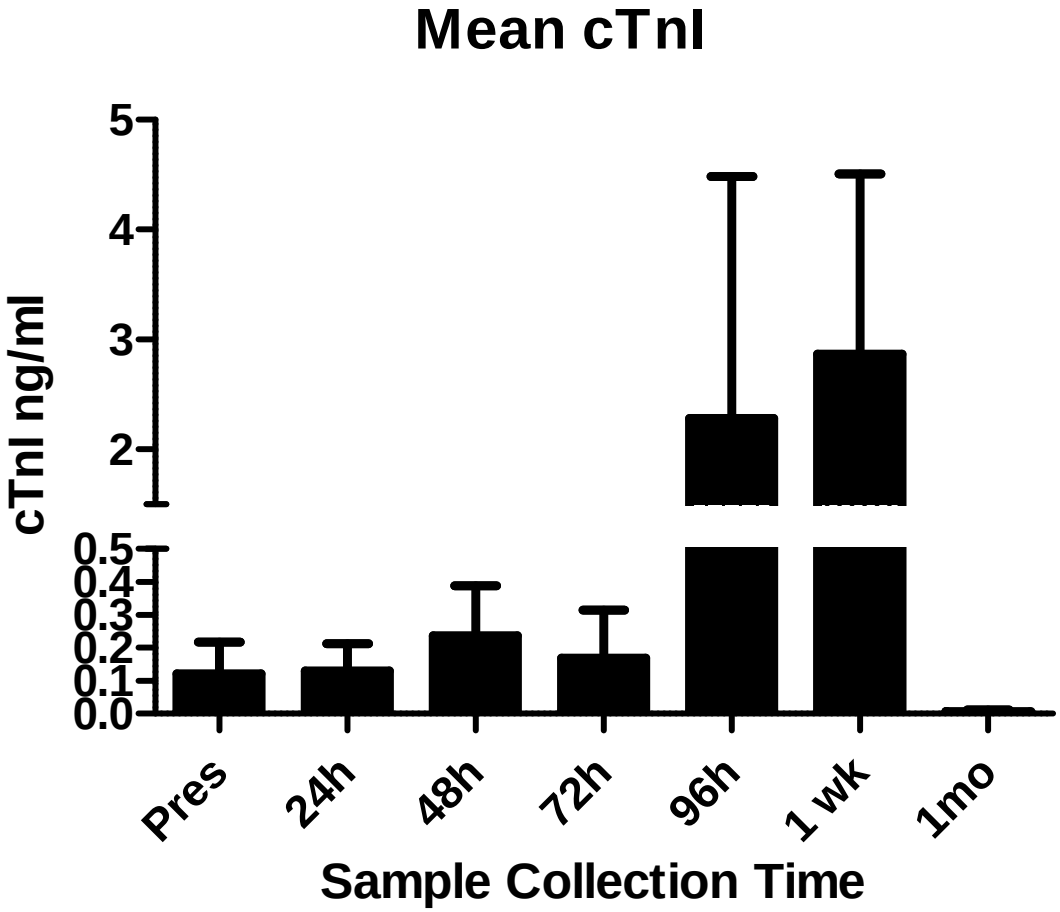
[21] Personal communication, Jennifer Taylor, Red Rock Biologics, Woodland, CA.

Table 4 – Characterization of arrhythmias in rattlesnake bitten and control horses

Arrhythmia	Bitten (n = 14)	Bitten + Inc. cTnI (n = 8)	Bitten + Norm. cTnI (n = 6)	Control (n=6)
APC	11	8	3	2
Sinus pause	7	4	3	0
Sinus tachycardia	2	1	1	0
Sinus arrest	2	1	1	0
AIVR	1	1	0	0
VPC	2	2	0	0
SVT	1	0	1	0
Sinus arrhythmia	1	1	0	0
Atrial fibrillation	1	1	0	0
2 or more of the above	8	6	2	0

APC = atrial premature contraction, AIVR = accelerated idioventricular rhythm, VPC = ventricular premature contraction, SVT = supraventricular tachycardia

Figure 4 : Mean cTnI of horses with increased cTnI (8) at each sample collection time point



DISCUSSION

A number of studies have utilized cTnI as a sensitive marker to document myocardial injury in the horse, (Divers, Kraus et al. ; Holbrook 2006; Holbrook 2008; Fennell and Forbes 2009; Kraus 2010; Durando 2011; Nostell 2011) and its use is becoming more common in the clinical evaluation of horses bitten by rattlesnakes. The cTnI from the rattlesnake-bitten horses was compared to the established normal range for the laboratory performing the assay. Cardiac troponin I has not been measured in horses with non-snake bite- associated puncture wounds. In order to determine with certainty that the increase in cTnI is associated with rattlesnake envenomation and not simply wound associated inflammation, cTnI would need to be measured in horses with similar wounds. Cardiac troponin I typically increases rapidly (within 2 hours), peaks around 12-48 hrs, and remains increased as long as the injury continues (Serra 2010). In humans experiencing an ischemic myocardial event, cTnI can remain increased for 10 days (Rajappa 2005). The half life of cTnI in the horse has been reported to be 1-2 hours (Divers, Kraus et al.). Three of 8 horses had steadily increasing cTnI up to the 1-week sampling. The longer cTnI is increased, the more indicative it is of irreversible damage (Fonfara 2010). An initial increase in cTnI can occur with mild damage because cytosolic troponin is released first, but a more marked and persistent increase is consistent with the release of structurally bound troponin proteins indicative of irreversible or ongoing myocardial damage (Wells 2008; Serra 2010). In dogs with heart disease, increased cTnI in 1 sample was not a good prognostic indicator but evaluating trends in subsequent samples was a useful prognostic tool. A downward trend in subsequent samples was a good prognostic indicator whereas persistently high concentrations of $\geq$1.0 ng/ml were indicative of a poor prognosis (Fonfara 2010). The peak cTnI concentration has been correlated with infarct size in dogs suggesting the magnitude of cTnI increase may be helpful in assessing the extent of myocardial damage (Fonfara 2010). The horse in our study with the highest cTnI died acutely 48 days post-bite. This horse was presented 5

days post-bite. Cardiac troponin I was measured on days 6-9 and concentrations ranged from 20.48 ng/ml to 56.58 ng/ml.

Timing of the peak in cTnI was variable and occurred between 12 hours and 1 month post envenomation (Figure 4). In 4 of 8 horses, the peak occurred at 7-30 days post-presentation. Although this was an unexpected finding, we suspect that cTnI appearance may be influenced by delayed venom absorption from damaged tissues. When horses are envenomated, the area around the bite site can have marked swelling, resulting in decreased blood flow to the area. This decreased perfusion may slow the absorption of venom into systemic circulation thereby postponing its cardiotoxic effects. Human snakebite victims can develop what is known as the recurrence phenomenon which describes a recrudescence of symptoms of envenomation up to 1 week after being bitten. These individuals were treated with anti-venom and signs of envenomation improved or disappeared and then recurred up to 1 week later (Boyer 2001; Seifert 2001).

Larger venom proteins may remain in circulation and be excreted, whereas smaller proteins may escape into the extravascular tissues and remain there for a longer period of time before being metabolized (Boyer 2001). Variable venom elimination may allow certain venom components to remain in the body longer, thus exerting their toxic effects at later time points in tissues such as the heart. Considering that 50% of the horses with biochemical evidence of cardiac injury demonstrated this delayed pattern of cTnI increase (7-30 days post-envenomation), clinicians should remain diligent in monitoring horses for myocardial damage after rattlesnake bite. Notably, others have documented horses with signs of cardiac disease 12 days to several weeks after envenomation (Lawler, Frye et al. 2008).

When statistically evaluated as a whole, rattlesnake bitten horses were not more likely to experience arrhythmias than normal horses hospitalized for Holter monitor data collection. This

could have been due to the small number of normal horses that were evaluated. It also could indicate that some arrhythmias noted in rattlesnake bitten horses are not necessarily caused by the rattlesnake venom but rather are a consequence of some factor associated with the bite such as stress or systemic inflammation. In order to better make this determination, a comparison should be made with Holter recordings evaluated on horses with similar circumstances such as puncture wounds not associated with rattlesnake envenomation. Rattlesnake bitten horses with increased cTnI were more likely to have arrhythmias than those with normal cTnI but arrhythmias were seen in horses with normal cTnI. Thus, a normal cTnI cannot rule out the development of cardiac irritability or electrical disturbance subsequent to envenomation. Arrhythmias ranged in severity from occasional atrial premature complexes to frequent atrial premature complexes and paroxysmal atrial fibrillation. Ventricular arrhythmias were found in 2 horses; both had markedly increased cTnI. The horse with the highest cTnI that was found acutely dead 48 days post-presentation did not have ECG abnormalities, but only 1 24-hour Holter reading was obtained on this horse 1 week post-presentation. In general, arrhythmias were mild and included occasional atrial premature contractions, 2 couplets and rare sinus pauses. Only 1 horse had an arrhythmia auscultated on physical examination, therefore continuous ECG monitoring may be necessary to detect intermittent arrhythmias after envenomation. Arrhythmias may not be evident immediately after rattlesnake envenomation. Horses with increased cTnI should receive long-term ECG follow-up to rule out the possibility of delayed development of arrhythmias. The clinical relevance of these arrhythmias should be determined by more extensive long term follow-up including echocardiography and exercise stress testing.

Although there are many potential mediators of the systemic inflammatory response, TNF-α has been specifically implicated as an important cytokine involved in rattlesnake envenomation (Moura da Silva, Laing et al. 1996). TNF-α primarily is produced by macrophages in affected tissues (Beutler and Grau 1993). It can be beneficial or harmful depending on the

amount produced and duration of time over which its production is sustained (Beutler and Grau 1993). High concentrations of TNF-α over prolonged periods of time have been shown to be detrimental to cardiomyoctes (Fernandez-Velasco, Ruiz-Hurtado et al. 2007). TNF-α production may be amplified after pit viper envenomation by the action of venom metalloproteinases converting TNF substrates into their biologically active form therefore allowing more TNF-α to be present in circulation (Moura da Silva, Laing et al. 1996). TNF-α has been shown to have hypotensive effects in rats envenomated with *Vipera aspis* (Szold, Ben-Abraham et al. 2003). Blocking the effects of TNF-α by administering anti-TNF-α antibody ameliorated cardiotoxic effects of venom in these rats (Szold, Ben-Abraham et al. 2003). Exposing rat ventricular myocytes to TNF-α resulted in electrophysiological changes (Fernandez-Velasco, Ruiz-Hurtado et al. 2007). TNF-α has been found to be detrimental in human cardiac patients with ischemia and reperfusion injury (Oral 1995). TNF-α causes endothelial cells to release many procoagulant factors that favor thrombosis and can result in disseminated intravascular coagulation (Beutler and Grau 1993). Additionally, TNF-α is directly cytotoxic and can create a vascular leak (Beutler and Grau 1993).

In these snake-bitten horses, there was a positive correlation between TNF-α and cTnI. Considering TNF-α has been shown to be cardiotoxic in other species, there could be a direct causal relationship between these variables in the horses in our study. Alternatively, the higher TNF-α and cTnI concentrations may merely reflect larger venom doses and these 2 markers may not be directly related. The urine venom ELISA results however do not necessarily support this hypothesis. Urinary excretion of venom appears to be highly variable among horses, and urine sample data sets were incomplete. Thus, we cannot rule out that increased markers of systemic inflammation (TNF-α) and myocardial injury (cTnI) reflect venom dosage.

We hypothesized that some horses may have pre-existing venom antibodies, which may be cardioprotective. We were unable to make this association. There are several possibilities for

the positive correlation between increased cTnI and peak venom antibody titers when cTnI was > 2 ng/ml and titers were > 5000. Higher venom antibody titers may indicate absorption of (and immune response to) a larger venom dose. As mentioned above however, more precise sampling times and a complete data set may be necessary to make that correlation. Higher venom antibody titers also may be simply indicative of an individual's more marked immunological response to venom. This marked immune response could be associated with immune-mediated damage to the heart as occurs in conjunction with other forms of myocarditis such as viral or parasitic myocarditis (Andrade, Andrade et al. 1994). Serum anti-venom titers did not persist long enough to conclude that horses maintain antibodies from natural exposure that provide long-term protection from subsequent envenomations.

Although venom was not detected in all bite site samples, this observation does not rule out envenomation. Depending on the amount of time that lapsed between the bite and presentation, bleeding or serous drainage could wash venom away from the wound or drying and dessication could occur, leading to negative results. The amount of venom at the bite site did not correlate with the degree of myocardial damage. In fact, in at least 1 horse the venom detected at the bite site was very high whereas no venom was detected in the urine and there was no increase in cTnI, indicating most of the venom was left on the skin and did not actually enter circulation.

Rattlesnake venom has been detected in the urine of a human rattlesnake bite victim (Ownby, Reisbeck et al. 1996). Excretion started at day 3 and continued until measurement was stopped at day 6 (Ownby, Reisbeck et al. 1996). In our horses, the earliest time point post-bite that venom was detected was within the first 24 hours and venom was detected in the highest number of horses (7/13) at 4 days. Venom was not detected in every sequential daily sample once it was initially detected, indicating that venom excretion may be intermittent. Because of this variable excretion, peak venom concentration could have been missed in some of our patients. Additionally, we had small numbers of horses with only 8 horses having increased cTnI.

Urine was not collected sequentially in all 8 horses making a statistical comparison difficult. To better compare peak urine venom concentrations with cTnI, more frequent sample collection will be required. Although it is clear from our results that a single sample is unlikely to be adequate to make an estimation of venom dose based on urine venom concentrations, additional research will be required to determine ideal sample collection timing.

In the area where these samples were collected, horses could have been bitten by *Crotalus viridis viridis* (Prairie rattlesnake), *Crotalus atrox* (Western Diamondback rattlesnake), *Sistrus milliarus streckerii* (Pigmy rattlesnake), or *Crotalus horridus horridus* (Timber rattlesnake). *Agkistrodon contortrix lacintus* (broad-banded copperhead) also is endemic in the areas where 3 of these horses resided. There are various reports in the literature of the occurrence of cardiac damage after snakebite in the horse (Fielding, Pusterla et al. ; Dickinson, Traub-Dargatz et al. 1996). The apparent discrepancy in mortality rates between these 2 studies may be influenced by the different snake species endemic to these regions. We cannot rule out the possibility that horses in this study that had myocardial damage (increased cTnI) were bitten by a different species of snake than those that did not have increased cTnI. In at least 1 study, cardiac tissue has been shown to be relatively resistant to the direct effects of the venom from 1 of the snakes endemic to our study areas (*Crotalus atrox*) (Posner, MacIntosh et al. 1981). Our venom assay was not able to differentiate among these species of snakes.

Treatment of all horses in this study was left to the discretion of the attending veterinarian. Most of the horses were treated with corticosteroids (13/15) and antimicrobials (13/15). The mouths of rattlesnakes have been shown to harbor a mixed population of bacteria, and although commonly accepted as standard of care in equine medicine, the use of antimicrobials in human snakebite victims is not deemed necessary (Singletary 2005). Corticosteroids have been used in the treatment of rattlesnake envenomation for decades (Russell 1980). An increased mortality in dogs treated with corticosteroids post-envenomation has been

reported (Segev, Ohad et al. 2008) but other studies have not shown the same results (Hackett 2002). Although there is no evidence that corticosteroids increase mortality in the horse, (Fielding, Pusterla et al. ; Dickinson, Traub-Dargatz et al. 1996) our treatment groups lacked sufficient size to make this comparison. Dexamethasone has been shown to inhibit TNF-α synthesis as has pentoxifilline (Han 1990). Additional investigation is needed to define the role of TNF-α in snake venom-mediated cardiac damage, but decreasing TNF-α production may prove beneficial in ameliorating the systemic effects of rattlesnake envenomation. This may give reason to reconsider the use of these drugs in the treatment of rattlesnake envenomation.

In conclusion, cardiac damage evidenced by increased cTnI occurs in horses bitten by rattlesnakes. Although this damage does not occur in all horses, repeated cTnI measurement is a valuable tool for detecting and monitoring cardiac damage. Continuous ECG recording may be useful in detecting snakebite-associated arrhythmias, particularly in those horses with increased cTnI. Snake bite-associated cardiac damage may be influenced by the envenomation dose and nature of the associated systemic inflammatory and immunologic responses, but we were unable to make these correlations in this study. The clinical outcome may be influenced both by individual variation in response to venom among horses as well as the individual snake species. Although arrhythmias in our study horses most frequently were mild, transient and did not cause clinical signs, more serious arrhythmias have been reported (Lawler, Frye et al. 2008) and long-term follow up is warranted. Due to the often delayed increase in cTnI in our study population, we recommend that horses be monitored closely for 1 week and then rechecked at later time points (weekly up to 6 weeks) to rule out the possibility of permanent cardiac damage or dysfunction secondary to the rattlesnake bite. In horses with clinical evidence of cardiac injury, echocardiography and exercise stress testing could be warranted.

RELEVANT FINDINGS

Cardiac abnormalities in this population of horses indicate that cardiac damage after rattlesnake bite is common. Rattlesnake bitten horses should be monitored for signs of cardiac damage and dysfunction. Long-term follow up should be encouraged to detect delayed cardiac dysfunction. The ability to prevent cardiac damage in rattlesnake bitten horses would be very valuable. A rattlesnake toxoid vaccine is marketed for the horse. If horses mount a robust immune response to this vaccine circulating antibodies may offer protection against the effects of rattlesnake envenomation.

CHAPTER V

ANTIBODY RESPONSE TO NATURAL RATTLESNAKE ENVENOMATION AND A RATTLESNAKE TOXOID IN HORSES

Submitted for Publication

ABSTRACT

Clinical response to natural rattlesnake envenomation in the horse is highly variable. Reasons for this variability are not clear. Previous venom exposure with production of anti-venom antibodies may account for some of the variability. Anti-venom antibody titers following rattlesnake bite in horses are not well documented. Furthermore, it is unknown whether the commercially available rattlesnake venom toxoid vaccine is capable of stimulating production of antibody titers similar to those produced during natural envenomation.

The first objective of this study was to measure antibody titers in horses bitten by rattlesnakes and compare them to antibody titers in horses vaccinated with the rattlesnake venom toxoid. The second objective was to compare antibody titers to the rattlesnake venom toxoid vaccine in pregnant versus non-pregnant mares and geldings.

Blood samples were collected from16 horses with clinical diagnosis of rattlesnake bite at presentation, 11 days and one month post-presentation. Serum was harvested and assayed for

anti-*Crotalus atrox* antibodies using an ELISA. 39 horses (12 pregnant mares, 13 non pregnant mares, and 14 geldings) were vaccinated using a *Crotalus atrox* venom toxoid in a series of three doses given intramuscularly 30 days apart. Blood was collected from the foals born to vaccinated mares prior to their first suckle and at 24 hours of age. Serum was harvested and antibodies were measured using an ELISA.

Horses bitten by a rattlesnake had significantly higher ($P<.0.004$) antibody titers compared to horses given the rattlesnake toxoid vaccine. There was no significant difference between antibody titers in pregnant versus non-pregnant mares and geldings that received the rattlesnake toxoid vaccine. There were no adverse effects in late gestation mares given the rattlesnake toxoid vaccine. Two mares had positive titers at foaling and both of their foals had positive titers at 24 hours of age.

Horses develop anti- *Crotalus atrox* antibody titers to the commercially available rattlesnake toxoid vaccine; however titers are not as high compared to natural envenomation. Whether or not antibodies derived from toxoid administration are protective against the side effects of natural envenomation has not been tested. The commercially available rattlesnake toxoid was safe when administered to late gestation mares and there is evidence that colostral transfer of venom antibodies does occur.

INTRODUCTION

Horses vaccinated for the commercial production of anti-venom have variable immune responses to venom (Sriprapat, Aeksowan et al. 2003). Venom from different species of snakes elicit different magnitudes of humoral immune response (da Silva and Tambourgi 2011). Crude venom and the isolated crotoxin from the South American Rattlesnake (*Crotalus durussis terifficus*), for example, have been shown to suppress the humoral immune response (da Silva and Tambourgi 2011) to venom. Additionally, the humoral immune response to snake venom

varies with the species of animal envenomated (Russell 1988). It is not known how these differing immune responses to venom affect the clinical outcome of envenomation; however, it has been shown that circulating anti-venom antibodies present at the time of or shortly after experimental envenomation are effective at decreasing the toxic effects of venom in mice (Rucavado and Lomonte 1996). Although antibody titers are frequently measured in horses used for the production of various anti-venoms, little is known about the venom antibody titers produced in horses after natural rattlesnake envenomation or the duration that antibody levels persist (Angulo, Estrada et al. 1997). Clinical and laboratory responses to natural rattlesnake envenomation vary in the horse and it is unknown whether varying immune responses play a part in this variability (Dickinson, Traub-Dargatz et al. 1996; Fielding, Pusterla et al. 2011; Gilliam, Holbrook et al. 2012).

Humans have an anamnestic response to snake venom (Theakston, Reid et al. 1981; Pe, Aye-Aye-Myint et al. 1995). In people bitten by the king cobra, there is evidence that the humoral immune response to repeated envenomations is greater, more effective at neutralizing venom effects and longer lasting than that of a single envenomation (Pe, Aye-Aye-Myint et al. 1995). This has also been shown to be true in Waorani Indians bitten by poisonous snakes endemic to the area where they live (Theakston, Reid et al. 1981). Following natural envenomation, the persistence of circulating antibodies is highly variable in humans. In a human bitten by the puff adder (*Bitis arietans*) antibodies were measurable out to 81 days post envenomation (Theakston 1985). In two patients bitten by the king cobra (*Ophiophagus hannah*) titers lasted approximately 8 weeks (Pe, Aye-Aye-Myint et al. 1995), and antibodies have persisted for 40 years in a patient after being bitten by a black-necked spitting cobra (*Naja nigricollis)* (Theakston, Pugh et al. 1981). Because people bitten multiple times often have more mild venom effects, vaccination against venom has long been attempted (Wiener 1960; Russell

1988). However, snake venoms seem to make poor immunogens and the duration of immunity is unpredictable (Wiener 1960; Glenn, Becker et al. 1970; Oliveira, Melo et al. 2007).

The purpose of this study was to measure venom antibody titers in horses bitten by rattlesnakes and to compare these titers with those administered a commercial rattlesnake toxoid vaccine. The immunologic response to vaccination in late gestation mares, and colostral transfer of antivenom antibodies in foals was also investigated.

MATERIALS AND METHODS

Study population and data collection

Sixteen horses with a clinical diagnosis of rattlesnake bite in the southwestern United States were enrolled in the study. There were 7 non-gravid mares and 6 geldings. The mean age was 4.67 years (median 4 years). The sex and age were not reported for 3 horses and one horse had sex but not age reported. Serum samples were collected at the time of presentation, 11 days and one month following presentation, and frozen at -80°C until analysis was performed. All horses had samples collected at presentation; however, not all horses were available for both the one week and one month samples. (Table 5)

Thirty-nine healthy adult horses that are part of the university research herd were enrolled in the toxoid vaccination study. These included, 12 late gestation mares (>270 days), 13 non-pregnant mares, and 14 geldings. Ages ranged from 4-20 years with a mean age of 13.2 years (median -14 years). Each horse received three doses (2ml) of a rattlesnake toxoid vaccine[22] in the pectoral muscle at 30 day intervals. Serum samples were obtained prior to administration of each vaccination and 30 days following the last vaccination. All horses were monitored for adverse vaccine reactions. Overall demeanor was observed and the vaccination site was palpated 24 hours after vaccination. A visual examination was performed on days 7, 14 and 21 after each

[22] Red Rock Biologics, California

vaccination. All late gestation mares were followed to parturition and the status of the foal at birth was noted. Serum was collected from foals prior to their first suckle and at 24 hours of age. Samples were not available for 3 foals. All serum samples were stored at -80C until assayed.

Venom antibody ELISA

Serum samples were assayed for antibodies against *Crotalus atrox* using standard ELISA techniques. Anti-equine alkaline phosphatase antibody[23] was used as a detection antibody. Plates were read at 405nm by spectrophotometer[24] and titers were calculated using a standard curve. Titers greater than 1:200 were considered positive according to venom neutralization studies performed by the vaccine manufacturing company[23].

DATA ANALYSIS

In order to correct for the lack of normality and homogeneous variances, the data were transformed with a square root function. A student's t-test was used to compare the venom antibody titers of horses naturally envenomated to those receiving the rattlesnake toxoid vaccine A Fisher's Exact test was used to assess differences in categorical titer levels of vaccinated late gestation pregnant mares versus non-pregnant vaccinated horses (mares and geldings).

RESULTS

Venom antibody titers were measured at presentation in sixteen horses with a clinical diagnosis of rattlesnake bite. Follow-up titers were measured at 11 days in 7 horses, one month in 4 horses, and both 11 days and one month in 4 horses. One horse was not available for a follow-up sample. Time after bite to initial presentation was <24 hours in 14 horses and 1 week in 2 horses. All horses that presented within 24 hours of the bite had negative titers upon presentation. Five

[23]Anti-equine alkaline phosphatase antibody, Sigma, St.Louis, MO

[24]THERMOmax microplate reader, Molecular Devices, Sunnyvale, CA

horses had negative titers at one week. Three of these five horses did not have one month samples available for assay. The two that had one month samples assayed had positive titers at one month post presentation. In horses where titers were measured at both one week and one month the highest titer was used for statistical comparisons. (Table 5)

Venom antibody titers were measured in 39 healthy research horses at four time points; prior to the first vaccination and 30 days after receiving each of three doses of the rattlesnake toxoid vaccine[1]. Peak titers were used for statistical comparisons. Horses bitten by a rattlesnake had significantly higher peak antibody titers than horses receiving the rattlesnake toxoid vaccine ($p<0.004$). (Figure 6) Ten of thirty-nine horses (25.6%) showed no response to the vaccine series. Two horses developed a peak titer 30 days after the first vaccination, 12 horses had a peak titer 30 days after the second vaccination and 15 horses developed peak titers 30 days after the third vaccination. (Figure 7) Thirty days after the last vaccination, 15 horses had increasing titers while 14 horses' titers were decreasing or unchanged. Twelve of the vaccinated horses were mares in late gestation and all of them gave birth to live, healthy foals. There was no difference in peak titers between non-pregnant horses (mares and geldings) and pregnant mares at any time point. Pre and post suckle serum samples were available from 9 foals born to vaccinated mares. The mares were at various stages in the vaccine series at foaling. Only two of the mares had positive titers prior to foaling. Both of the foals born to these mares had positive post suckle titers. (Table 6)

All vaccinated horses were monitored for adverse vaccine reactions. Generalized enlargement of the pectoral muscle was noted in three horses and a firm knot at the injection site was noted in two horses. The injection site reactions in these five horses were monitored daily and they all resolved within 21 days without treatment. No changes in attitude or appetite were noted in any of the vaccinated horses.

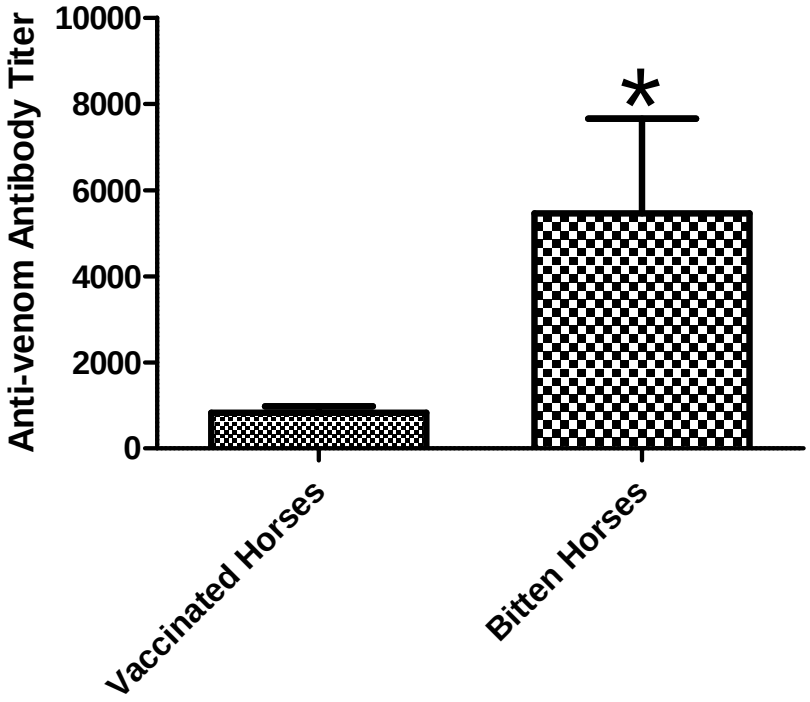
Table 5: Sample timing and anti-venom antibody titers of rattlesnake bitten horses.

Horse	Time Post-Bite of Sample Collection*	Antivenom Antibody Titer
1	0	130
	11 days	1370
	1 month	515
2	0	105
	11 days	140
	1 month	245
3	0	100
	11 days	825
4	0	95
	1 month	2825
5	0	100
	1 month	5860
6	0	85
	1 month	9975
7	0	110
	11 days	120
8	0	120
	11 days	125
	1 month	1435
9[a]	1 week	130
10	0	105
	11 days	5570
11	0	120
	11 days	295
12[a]	0	4250
	11 days	17060
13	0	135
	11 days	925
	1 month	1620
14	0	120
	11 days	170
15	0	115
	1 month	6130
16	0	95
	1 week	33600

*Time 0 was presentation

[a]Horses 9 and 12 presented one week after being bitten

Figure 5: Peak anti-venom antibody titers in vaccinated versus bitten horses.



*P<0.0004

Figure 6: Number of horses with peak anti-venom antibody titers 30 days after each vaccine dose.

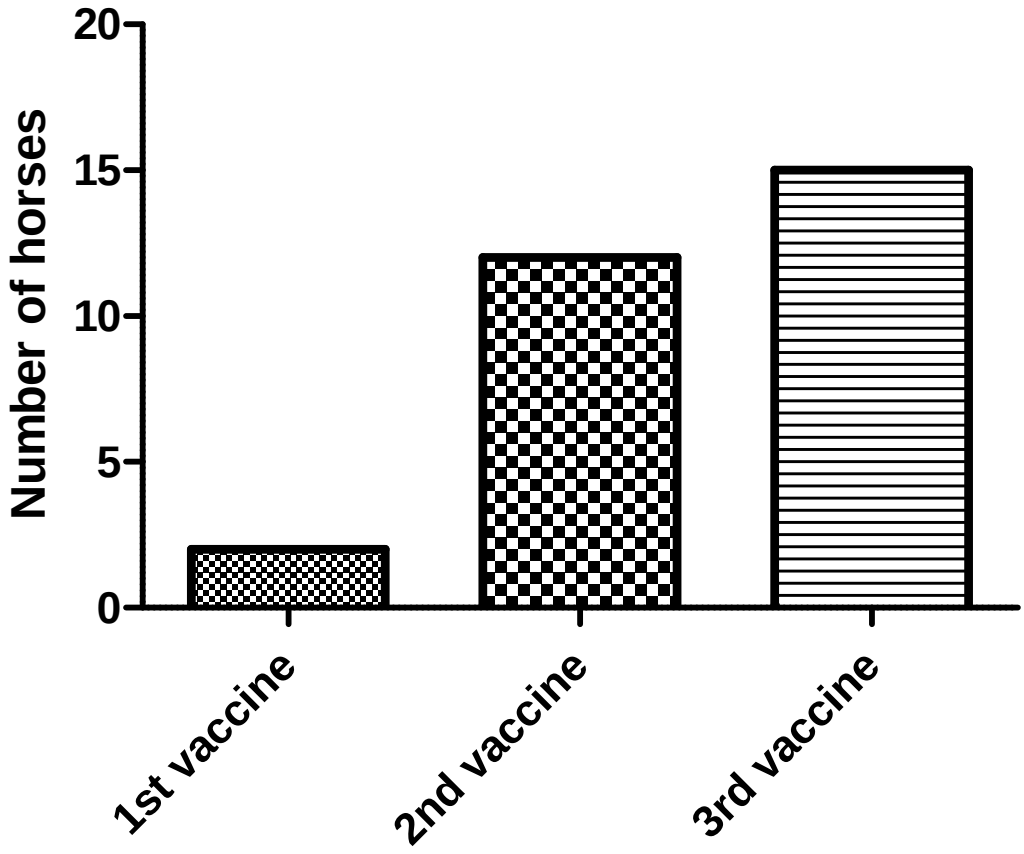


Table 6: Pre and 24 hour post suckle anti-venom antibody titers in foals born to vaccinated mares.

Horse	**# of vaccines given prior to parturition**	**Titer at parturition**	**Foal's Pre Suckle Titer**	**Foal's 24 hr Post Suckle Titer**
28	3	120	95	125
30	2	375	105	280
31	3	200	110	185
32	2	125	75	175
34	1	715	75	745
35	1	100	80	95
36	1	125	90	140
37	1	195	90	115
39	2	125	105	105

*Grey boxes indicate mares with positive titers at parturition.

DISCUSSION

Horses have long been used for the production of commercial anti-venoms. The production of antibodies in horses vaccinated with crude venom and venom components is well studied (Theakston 1989; Freitas, Fortes-Dias et al. 1991; Angulo, Estrada et al. 1997; Sriprapat, Aeksowan et al. 2003; Lavonas 2012); however, the antibody response of horses to natural rattlesnake envenomation is not well documented. One goal of this study was simply to document antibody titers in naturally envenomated horses and to document the presence or absence of pre-existing venom antibody titers. None of the horses in this study had antibody titers within 24 hours of being bitten. Documentation of previous envenomation was not available for these horses. The absence of positive titers less than 24 hours post bite in these horses was most likely because this was their first exposure to rattlesnake venom. Alternatively, the time since previous exposure to rattlesnake venom could have exceeded the half life of the venom antibodies. There are varying reports of the duration of venom antibody titers in humans (Theakston, Pugh et al. 1981; Theakston, Reid et al. 1981; Theakston 1985; Domingos, Cardoso et al. 1990; Pe, Aye-Aye-Myint et al. 1995) following natural snake envenomation (8weeks – 40 years). In goats experimentally envenomated with *Crotalus atrox* venom, antibodies were short lived (~60 days) (Glenn, Becker et al. 1970). Horses used for snake anti-venom production must be repeatedly immunized in order to maintain adequate titers (Guidlolin, Marcelino et al. 2010). Repeated immunization protocols range from every 2 days (Freitas, Fortes-Dias et al. 1991) to every 2 weeks (Guidlolin, Marcelino et al. 2010) and there is a large variation in response among individual horses (Sriprapat, Aeksowan et al. 2003). Therefore, it is possible that venom antibody titers following natural envenomation do not persist for prolonged periods of time. Three horses had negative post-envenomation titers; however they did not have a sample drawn one month post-envenomation. There are a couple possible reasons for these negative titers in the face of clinical signs of rattlesnake envenomation. It is possible, and most likely, that this was these horses' first exposure to rattlesnake venom and 11 days was too early to detect a rise in titer. The

immune response to venom is dependent upon the structure and molecular mass of the venom toxins and their relative abundance in venom, the dose of venom administered and the host's ability to recognize the venom as foreign (Leon, Sanchez et al. 2011). The host's ability to recognize the venom as foreign may depend on the route by which it enters the body, the animal's ability to process the venom toxins and the individual animal's genetic background (Leon, Sanchez et al. 2011). In humans bitten for the first time by *Bothrops jararaca* the increase in IgG occurred at 18 days post envenomation (Domingos, Cardoso et al. 1990), while those bitten two or more times develop titers by day three (Domingos, Cardoso et al. 1990). It is also possible that these horses received a dry bite or a very small dose of venom that was enough to cause local inflammation but not enough to result in a humoral immune response. During the production of anti-venom it is frequently noted that there can be marked differences in individual animals' response to venom (Leon, Sanchez et al. 2011; Villalta, Pla et al. 2012). It is possible that although these horses were envenomated they are not capable of mounting a humoral immune response against the venom. Finally, there is a possibility that the assay used in this study was not able to detect venom antibodies to the species of rattlesnake that had bitten these horses. These horses came from areas of Oklahoma and Texas where several rattlesnake species reside along with the broad banded copperhead (*Agkistrodon contortrix laticinctus*) and the water moccasin (*Agkistrodon piscivorus*). None of the snakes that bit these horses were positively identified. Although the assay used in this study was designed to detect antibodies against Western diamondback rattlesnake (*Crotalus atrox)* venom, cross reactivity to multiple rattlesnake venoms has been well demonstrated. Strength of cross reactivity varies depending on the species being compared thus if a horse was bitten by a snake with weak cross reactivity with *Crotalus atrox* this assay may not detect a positive anti-venom antibody titer. While this is possible, it is unlikely to be the cause of the negative titers (Ownby and Colberg 1990).

A second aim of this study was to compare the titers of the naturally envenomated horses to those of horses vaccinated with a commercially available rattlesnake toxoid vaccine. Titers in the naturally envenomated horses were significantly higher than those in the vaccinated horses. This could be due to a higher antigen load in the naturally envenomated horses or due to a reduced immune response to the detoxified venom. One primary disadvantage of immunization with a detoxified venom is the risk of losing epitopes that are important in the immunogenicity of the venom (Leon, Sanchez et al. 2011). Approximately 25% of the vaccinated horses did not respond to the rattlesnake vaccine. This is not surprising based on the individual animal variability that is seen in animals that are vaccinated with crude venom for antibody production (Guidlolin, Marcelino et al. 2010; Leon, Sanchez et al. 2011). The anamnestic response to this vaccine was also quite variable considering approximately half of the horses had increasing titers 30 days after the third vaccine booster while half had decreasing titers. The manufacturer's recommendation is to give boosters every 6 months after the initial series of three doses given 30 days apart. It may be prudent to consider revising this dosing schedule to booster the animal more frequently during the rattlesnake season and not administer the vaccine during times when the horse is highly unlikely to encounter a rattlesnake in order to maintain peak titers during peak exposure times.

Thirteen of the horses that were vaccinated were pregnant mares. Pregnancy has been shown to down regulate the Th1 response of the immune system and shift it more towards a Th2 response (Noronha and Antczak 2010). This would typically promote a more robust humoral immune response however we did not detect a difference in the overall antibody titers in the pregnant mares versus the non-pregnant horses (mares and geldings). Pre and post suckle serum samples were only available on foals from 9 of the 12 vaccinated mares and only 2 of these mares had positive titers at parturition. The foals from the 2 mares with positive titers had negative pre-suckle titers and a positive titer at 24 hours of age. These results suggest that colostral transfer of

venom antibodies produced in response to the toxoid vaccine may be possible. All the foals were born healthy and no adverse vaccine reactions were noted in the mares indicating the vaccine is safe to be given to late gestation mares.

We hypothesized that the variability in clinical response to rattlesnake envenomation in horses may be due to pre-existing antibody titers in individuals that had been previously bitten. The absence of pre-existing antibodies in this population of horses indicates the clinical variability was not due to pre-existing antibody titers; however this may not be true in all populations. In humans experiencing snake bite there is evidence that pre-existing titers may protect against mortality but not against the local venom effects (Wiener 1961; Theakston, Reid et al. 1983; Isbister, Halkidis et al. 2010). A person bitten by a death adder (*Acanthophis* sp.) that had a pre-existing antibody titer from a previous bite did not experience the typical neurotoxic signs although they experienced the local tissue effects of the venom (Isbister, Halkidis et al. 2010). Horses bitten by rattlesnakes typically experience significant local tissue effects but can also have systemic effects including cardiac damage (Dickinson, Traub-Dargatz et al. 1996; Fielding, Pusterla et al. 2011; Gilliam, Holbrook et al. 2012). Similar to this human case, pre-existing antibodies whether from vaccination or natural envenomation may not prevent local tissue effects but may have protective effects on other systemic effects such as cardiotoxicity. In most cases of envenomation the onset of the toxic effects of the venom are extremely rapid and antibodies would have to be in circulation at the time of envenomation to make a difference (Wiener 1961), however, in some horses that are bitten by rattlesnakes evidence of cardiotoxicity is not seen for several days after envenomation (Gilliam, Holbrook et al. 2012). The reason for this delay is unknown; however it has been hypothesized that it could be due in part to a delay in venom release from the tissues at the bite site. Pre-existing antibodies and an anamnestic response to venom exposure may be particularly helpful in these cases. Venom neutralization is not an all or none phenomenon, any amount of venom that is neutralized is effectively removed

from the circulation and therefore there is less to exert toxic effects on the animal. Interestingly, three of the 39 horses vaccinated with the rattlesnake toxoid vaccine had positive titers prior to receiving the vaccine. Each of these horses had what would be considered a robust response to the vaccine with titers greater than 1000. These horses were donated to our university and no history could be gathered on them as far as previous rattlesnake exposure. It is likely that they had been previously bitten; however, we could not rule out the possibility of a non-specific cross reactivity whereby antibodies not specific to rattlesnake venom reacted with the venom in this ELISA.

Antibodies that develop as a result of natural envenomation may be functionally distinct from those produced in response to a rattlesnake toxoid vaccine. As previously mentioned one major disadvantage to immunization with detoxified venom is the risk of losing important epitopes and therefore having decreased immunogenicity. Others have demonstrated that this can result in the inability of antibodies made against detoxified venom to neutralize native venom (Leon, Sanchez et al. 2011). However, studies performed during the licensing of the product we used for vaccination demonstrated venom neutralization with serum from horses vaccinated with the rattlesnake toxoid vaccine (unpublished data). Challenge studies in vaccinated horses have not been performed.

In conclusion, while horses develop anti- *Crotalus atrox* antibody titers to the commercially available rattlesnake toxoid vaccine; they are not as high as those that develop after natural rattlesnake envenomation. Individual horse's humoral immune responses to the vaccine and natural envenomation varied greatly. The commercially available rattlesnake toxoid vaccine was safe when administered to late gestation mares and there is evidence that colostral transfer of venom antibodies will occur. Based on existing literature, circulating titers may offer some protection against the systemic effects of envenomation; however, the ability of these titers to protect horses from the adverse effects of envenomation has not been tested *in vivo*.

CHAPTER VI

DISCUSSION

The first aim of this study was to develop an ELISA that could detect rattlesnake venom in equine biologic samples as there are no previous reports of venom detection in the urine or at the bite site of horses bitten by North American rattlesnakes. Colorimetric techniques did not produce an adequately sensitive assay, therefore fluorescence was used. The fluorescent assay was successful in detecting venom in urine and at the bite site of horses bitten by rattlesnakes. The sensitivity of the assay was 1 ng/ml. Our sample timing was inadequate to get an accurate estimate of venom dosage in these horses. If the ideal sample to detect maximum venom excretion can be better defined in the horse in a more controlled research setting then this assay could be used to detect venom and give an estimated dose. Anti-venom therapy is expensive and often cost prohibitive in the horse. In humans bitten by cobras determining the amount of venom in circulation was beneficial in guiding the use of anti-venom (Hung, Liau et al. 2003). Knowing the approximate dose of rattlesnake venom that a horse received may assist in determining whether or not anti-venom therapy is necessary.

The ideal goal of development of an assay to detect rattlesnake venom in equine samples would be to have a stall side test that could detect the amount of venom the horse received as well

as the species of rattlesnake that bit the horse. Anti-venoms that were readily commercially available were used in our study and contained antibodies to *Crotalus adamanteus, Crotalus atrox, Crotalus scutulatus scutulatus, Agkistrodon piscivorus, Crotalus durissus terrificus,* and *Bothrops atrox*. Cross reactivity does occur amongst rattlesnake species (Minton, Weinstein et al. 1984; Perez, Garcia et al. 1984; Minton 1987; Ownby and Colberg 1987; Berger and Bhatti 1989); therefore, although antibodies to all species endemic to the study area were not included in the polyvalent anti-venoms used in this ELISA, detection should still occur. Cross reactivity has been demonstrated between *Crotalus viridis viridis, Crotalus horridus horridus, Crotalus atrox* and *Crotalus adamanteus* (Minton 1987; Ownby and Colberg 1990; Li and Ownby 1992).

The use of polyvalent anti-venoms in this assay makes determining the species of snake impossible. A new direction of venom research is venomics, the use of proteomic techniques to further analyze snake venom. This technology has been successful in gaining detailed protein analysis of different snake venoms using mass spectrometry (LoMonte, Rey-Suarez et al. 2012). The use of this technology may be beneficial in detecting toxins that are specific to certain species of rattlesnakes so that more species specific assays can be developed.

A second aim of this study was to determine if cardiac damage, as evidenced by increased cTnI and ECG abnormalities, is a common occurrence in horses bitten by rattlesnakes. Forty percent of the horses in our study experienced increased cTnI and seventy percent had electrocardiographic abnormalities. This was confirmation that cardiac damage following rattlesnake bite in the horse is not uncommon and should be investigated.

A third aim was to investigate potential causes of the cardiac damage that follows rattlesnake envenomation in the horse. We wanted to investigate three major areas; venom dose, TNF-α concentrations and venom antibody titers. We hypothesized that the higher the venom dose that a horse received the more likely it would be to have cardiac damage. We were unable

to accept this hypothesis due to a lack of uniformity in sample timing and small study numbers. As mentioned above, to further investigate this hypothesis, a more controlled study environment will be required. An ideal situation for defining venom excretion in the horse would be to administer venom experimentally and then collect serial samples to determine ideal urine sample timing for venom detection. The systemic illness caused by envenomation is a concern in experimental envenomations. An ideal dosing scheme that provided enough venom for detection but not enough to cause systemic illness would need to be defined.

Based on the known affects of TNF-α concentrations on the heart in other species (Fernando-Martinez, Ruiz-Hertado et al. 2007, Szold, Ben-Abraham et al 2003, Mann 2001) as well as the ability of venom metalloproteinases to increase TNF-α activation (Moura da Silva, Laing et.al. 1996), we hypothesized that the cardiac damage following rattlesnake venom poisoning in the horse could be due to increased TNF-α concentrations. We were able to show a positive correlation between TNF-α and cTnI. This discovery may lead to different treatment options for rattlesnake bite in the horse. Treatments are currently centered on anti-inflammatories and supportive care. Although the use of steroids in the treatment of rattlesnake envenomation is controversial, their use may be reconsidered for their effect in decreasing TNF-α synthesis (Han, Thompson et.al. 1990). Pentoxifylline is a drug that is commonly used in equine medicine and it has been show to decrease TNF-α synthesis as well (Han, Thompson et.al. 1990). It may be a good adjunct therapy in rattlesnake envenomation. TNF-α inhibitors would likely be cost prohibitive in the horse and the pharmacokinetics have not been defined. TNF-α inhibitors are not without risk. A review of the human literature indicates that long term use of TNF-α inhibitors can lead to serious side effects including serious bacterial, viral, and fungal infections, a possible increased risk for development of lymphoma, worsening of clinical signs and pathology in patients with congestive heart failure and others (Lin, Ziring et.al. 2008). The adverse effects noted in patients with heart disease may make it an undesirable treatment in

horses with potential cardiac damage; however, in the earlier stages of disease in a rat model of volume overload TNF-α inhibitors have been shown to attenuate adverse myocardial remodeling (Jobe, Melendez et.al. 2009). If TNF-α inhibitors are used in the treatment of rattlesnake bite they would most likely be beneficial in the acute phase of the disease and not be required long term which may alleviate concerns over the adverse effects reported with use of this class of drugs.

Finally, in investigating a cause for cardiac damage following rattlesnake envenomation, we hypothesized that horses that had been bitten previously and therefore had pre-existing anti-venom antibody titers would be protected against the cardiotoxic effects of rattlesnake venom. We were unable to accept this hypothesis as none of our horses had pre-existing antibody titers.

With anti-venom being the treatment of choice for snake envenomation it would make sense that anti-venom antibodies present at the time of envenomation would be protective against the adverse effects of rattlesnake envenomation. A rattlesnake toxoid vaccine is commercially available for horses and we hypothesized that horses would produce anti-venom antibody titers to this vaccine similar to those produced with natural envenomation. Although the horses in our study did produce antibody titers to the rattlesnake toxoid vaccine, naturally envenomated horses had significantly higher titers. Of interest was that antibody titers to the rattlesnake toxoid vaccine did not appear to be long lived with half of the horses having declining titers one month after their last booster. This finding could explain the absence of pre-existing anti-venom antibody titers in our naturally envenomated horses. The half life of anti-venom antibodies has been found to be variable in other species and perhaps is short in the horse (Theakston, Pugh et al. 1981; Theakston, Reid et al. 1981; Theakston 1985; Domingos, Cardoso et al. 1990; Pe, Aye-Aye-Myint et al. 1995, Glenn, Becker et al. 1970). *In vitro* venom neutralization studies have been performed using serum from horses vaccinated with the rattlesnake toxoid vaccine; however, challenge studies have not been done with the rattlesnake toxoid vaccine to determine if

vaccinated horses experience less deleterious effects. A study of this type will again require careful consideration of the toxic effects of rattlesnake venom when formulating a challenge dose.

In conclusion, an assay was developed for the detection of rattlesnake venom in equine biological samples. The use of this assay in a clinical setting requires further investigation. Cardiac damage evidenced by increased cTnI occurs in horses bitten by rattlesnakes. Although this damage does not occur in all horses, repeated cTnI measurement is a valuable tool for detecting and monitoring cardiac damage. Continuous ECG recording may be useful in detecting snakebite-associated arrhythmias, particularly in those horses with increased cTnI. Snake bite-associated cardiac damage may be influenced by the envenomation dose and nature of the associated systemic inflammatory and immunologic responses, but we were unable to make these correlations in this study. The clinical outcome may be influenced both by individual variation in response to venom among horses as well as the individual snake species. Although arrhythmias in our study horses most frequently were mild, transient and did not cause clinical signs, more serious arrhythmias have been reported (Lawler, Frye et al. 2008) and long-term follow up is warranted. Due to the often delayed increase in cTnI in our study population, we recommend that horses be monitored closely for 1 week and then rechecked at later time points (weekly up to 6 weeks) to rule out the possibility of permanent cardiac damage or dysfunction secondary to the rattlesnake bite. In horses with clinical evidence of cardiac injury, echocardiography and exercise stress testing could be warranted. These findings enhance our knowledge of rattlesnake envenomation in the horse and open the doors for further investigation.

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VITA

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